Dkt. 43016-D/JPW/MAF

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Philip Livingston and Friedhelm Helling

U.S. Serial No.: 08/477,147 Group Unit: 1645

Filed : June 7, 1995 Examiner: P. Duffy

For : GANGLIOSIDE-KLH CONJUGATE VACCINE PLUS QS-21

Rule 129a w/Recon Linda 10/17/02

1185 Avenue of the Americas
New York, New York 10036
October 2, 2002

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

COMMUNICATION REQUESTING CONSIDERATION ON THE MERITS OF A SECOND SUBMISSION AFTER FINAL REJECTION WITH WITHDRAWAL OF FINALITY UNDER 37 C.F.R. §1.129(a) AND PETITION FOR A FOUR MONTH EXTENSION

This Communication is submitted pursuant to the provisions of 37 C.F.R. §1.129(a) to request consideration on the merits of an Amendment In Response To October 5, 2001 Final Office Action and Supplemental Information Disclosure Statement Submitted as a Second Submission, attached hereto as **Exhibit A** and withdrawal of the finality of the October 5, 2001 Office Action.

On October 5, 2001 the United States Patent and Trademark Office issued a Final Office Action in connection with the above-identified application. A response to the October 5, 2001 Office Action was due January 5, 2002. On April 5, 2002 Applicants' attorneys filed a Petition For A Three Month Extension of Time, and a Notice of Appeal From The Examiner's Decision To The Board of Patent Appeals and Interferences. A Brief on Appeal was therefor due June 5, 2002. Applicants hereby petition for a four month

extension of time for filing their response in this case. Applicants have previously established small entity status, which is still proper. The required fee for a four month extension of time for a small entity is SEVEN HUNDRED TWENTY DOLLARS ($720.00) and under 37 C.F.R. §1.17(r) the fee for filing a submission after final rejection is THREE HUNDRED SEVENTY DOLLARS ($370.00). Applicants therefore enclose a check in the amount of ONE THOUSAND NINETY DOLLARS ($1090.00) which covers these fees. Accordingly, the Amendment in response to October 5, 2001 Final Office Action and Supplemental Information Disclosure Statement Submitted as a Second Submission, attached hereto as Exhibit A, is now due October 5, 2001. Since October 5, 2002 is a Saturday, however, the response is due under 37 C.F.R. §1.7 on October 7, 2002, i.e., the next succeeding business day which is not a Saturday, Sunday or a Federal Holiday. This Communication and the attached Amendment are thus being timely filed.

Under 37 C.F.R.§1.129(a) applicants in an application that has been pending for at least two years as of June 8, 1995, taking into account any reference made in such application to any earlier filed application under 35 U.S.C.§120, 121 and 365(c), are entitled to have a Second Submission entered and considered on the merits after final rejection if the Second Submission and the fee set forth in 37 C.F.R.§1.17(r)are filed prior to the filing of an appeal brief and prior to the abandonment of the application. The present submission is a Second Submission in that a first such Submission Under 37 C.F.R.§1.129(a) was filed January 21, 2000 in response to a December 21, 1998 Final Office Action.

For its earliest filing date the subject application claims priority of U.S. Serial No. 08/009,268 filed January 22, 1993. Therefore, taking into account the priority reference, this application has been pending for at least two years as of June 8, 1995. This Communication is prior to the filing of an appeal brief and prior to the abandonment of the subject application. Accordingly, the Amendment In Response To October 5, 2001 Final Office Action and Supplemental Information Disclosure Statement Submitted as a Second Submission Under 37 C.F.R.§1.129(a) is being timely filed.

The fee under 37 C.F.R. for consideration and entry of a second submission after a final rejection is, as noted, $370.00. As stated hereinabove, applicants enclose herewith a check for $1,090.00 which includes this fee.

Under 37 C.F.R.§1.129(a) the finality of the final rejection is automatically withdrawn upon the timely filing of the Second Submission and the payment of the fee set forth in §1.17(r). Accordingly, applicants respectfully request that the finality of the October 5, 2001 Final Office Action be withdrawn and the Amendment in Response To October 5, 2001 Final Office Action and Supplemental Information Disclosure Statement be considered as a Second Submission.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys incite the Examiner to telephone at the number provided below.

No fee, other than the enclosed $1,090.00 fee which includes the $720.00 fee for a four month extension of time and the $370.00 fee under 37 C.F.R.§1.17(r) for filing this submission, is deemed necessary in connection with the filing of this Communication. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

Mark A. Farley

John P. White
Registration No. 28,678
Mark A. Farley
Registration No. 33,170
Attorneys for Applicants
Cooper & Dunham, LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Mark A. Farley 10/2/02

John P. White Date
Reg. No. 28,678
Mark A. Farley
Reg. No. 33,170

Fkt. 43016-D/JPW/MAF

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Philip Livingston and Friedhelm Helling

U.S. Serial No.: 08/477,147 Group Unit: 1645

Filed : June 7, 1995 Examiner: P. Duffy

For : GANGLIOSIDE-KLH CONJUGATE VACCINE PLUS QS-21

1185 Avenue of the Americas
New York, New York 10036
October 2, 2002

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

AMENDMENT IN RESPONSE TO OCTOBER 5, 2001 FINAL OFFICE ACTION AND SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT SUBMITTED AS A SECOND SUBMISSION

This Amendment is submitted in response to an October 5, 2001 Final Office Action issued by the United States Patent and Trademark Office as a second submission pursuant to a Communication Requesting Withdrawal of Finality under 37 C.F.R. 1.129(a) of the October 5, 2001 Final Office Action. Accordingly, this Amendment is being timely filed.

Please amend the subject application as follows:

In the claims:
Please amend claim 109 as follows:

A marked-up version of the amended claim, wherein the deleted material is in brackets and the inserted material is underlined, is attached hereto as **Exhibit 1**.

--109. (Amended) A composition which comprises:

a) a conjugate of i) a ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising an altered sphingosine base to ii) an immunogenic protein-based carrier comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja Saponaria Molina tree; and

c) a pharmaceutically acceptable carrier;

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject,

wherein the ganglioside derivative is a derivative of a ganglioside selected from the group consisting of GM2, GM3, GD2, GD3 lactone, O-acetyl GD3 and GT3; and wherein the immunogenic protein-based carrier is derived from a protein selected from the group consisting of malaria T-cell epitope, an outer membrane protein of Neisseria meningitidis, cationized bovine serum albumin, Keyhole Limpet Hemocyanin, polylysine and human serum albumin;

wherein in the conjugate the ganglioside derivative is covalently bound to the immunogenic protein-based carrier by a stable amine bond between the C-4 carbon of the altered sphingosine base of the altered ceramide portion of the

ganglioside derivative and the nitrogen of the ε-aminolysyl group of the immunogenic protein-based carrier.--

REMARKS

Claims 109-122 are pending in the application. Applicants acknowledge the Examiner's statement in ¶1 on p.2 of the Office Action, i.e., that claims 102-108 were previously pending and not claims 102-120 as stated in the Amendment filed July 17, 2001. The Examiner further stated that claims 102-108 were canceled and the new claims (misnumbered as nos. 121-134) submitted with the response of July 23, 2001 have been renumbered as claims 109-122 respectively. Applicants apologize for any confusion caused by the misnumbering of new claims 109-122 as nos. 121-134.

Claim 109 has been amended, as discussed below, to more clearly define the conjugation between the ganglioside derivative and the immunogenic protein based carrier. These amendments are completely supported by the application as originally filed, and in particular by Figure 1; page 5, lines 4-7; page 32, lines 13-20; and page 65, lines 9-15. Accordingly there is no issue of new matter. Entry of the Amendment into the file of the application such that claims 109-122, as amended, will be pending is therefore respectfully solicited.

Obviousness-Type Double Patenting Rejection

The Examiner provisionally rejected claims 109-122 as being unpatentable due to obviousness-type double patenting over claims 65-71 and 77 of copending Application No. 08/477,097. The Examiner stated, in regard to this rejection, that although the conflicting

claims are not identical, they are not patentably distinct from each other because they all claim conjugates by means of conjugating proteins to gangliosides through the ceramide portion and thus the particular method species drawn to GM2 or GM3 claimed in the copending application would anticipate the instant genus composition claims.

The Examiner stated that Applicants argue that the provisional rejection should be allowed to drop and the instant claims should be allowed to issue, pursuant to MPEP Section 804. The Examiner went on to state, however, that since the instant claims are not allowable, the provisional double patenting rejection is maintained for the reasons already made of record.

The Examiner has additionally provisionally rejected claims 109-122 due to obviousness-type double patenting over claims 66-72 of copending Application No. 08/475,784. The Examiner stated that although the conflicting claims are not identical they are not patentably distinct from each other because they all claim conjugates by means of conjugating proteins to gangliosides through the ceramide portion and thus the particular method species claimed in the copending application would anticipate the instant genus composition claims.

The Examiner additionally stated that Applicants argue that the provisional rejection should be allowed to drop and the instant claims should be allowed to issue pursuant to MPEP Section 804. The Examiner went on to state, however, that since the instant claims are not allowable, the provisional double-patenting rejection is maintained for the reasons already made of record.

Further to the above, claims 109-122 are provisionally rejected as being unpatentable for obviousness-type double patenting over claims 80-86 and 92-96 of copending application No. 08/196,154. As with regard to the above-discussed provisional double patenting rejections, the Examiner stated that although the conflicting claims are not identical, they are not patentably distinct from each other because they all claim conjugating proteins to gangliosides through the ceramide portion and thus the particular method species claimed in the co-pending application would anticipate the instant genus composition claims.

The Examiner further stated that Applicants had argued that the provisional rejection should be allowed to drop and the instant claims be allowed to issue, pursuant to MPEP Section 804. The Examiner went on to state, however, that since the instant claims are not allowable, the provisional double patenting rejection is maintained for the reasons already made of record.

The Examiner's provisional double patenting rejections are respectfully traversed. As noted in the Office Action, Applicants have argued in their prior response that the provisional double-patenting rejection should be allowed to drop, and the presently pending claims should be permitted to issue, pursuant to MPEP §804. The subject MPEP section, in discussing provisional double patenting rejections of the type raised in the present application, stated that the:

> 'provisional' double patenting rejection should continue to be made by the Examiner in each application as long as there are

> conflicting claims in more than one application, unless that 'provisional' double patenting rejection is the only rejection remaining in one of the applications. If the 'provisional' double patenting rejection in one application is the only rejection remaining in the application the Examiner should then withdraw that rejection and permit the application to issue as a patent, thereby converting the 'provisional' double patenting rejection in the other application into a double patenting rejection at the time one application issues as a patent. (emphasis supplied by Applicants).

The Examiner responded to the argument by Applicants based on MPEP §804 by pointing out that, upon the mailing of the Final Office Action, the 'provisional' double patenting rejection is not the only rejection remaining in the application. That is to say that there is, as set forth in the Final Office Action, a §112, first paragraph, and a §103(a) rejection of claims 109-122 of the application. The Examiner has thus stated that (as noted above), "Since the instant claims are not allowable, the provisional double patenting rejection is maintained for reasons already made of record".

In response to the Examiner's above-quoted statement, Applicants submit that for the reasons set forth below, the amendments made herein to independent claim 109 are believed to overcome both the §112 and the §103 rejection of that claim, as well as the corresponding rejection of those claims which depend from claim 109(i.e., remaining claims 110-122), which rejections should therefore be withdrawn. Following such withdrawal, the only remaining rejection would be the obvious-type double patenting

rejection. In accordance with MPEP §804, therefore, the double patenting rejection should then also be withdrawn to permit the present application to issue as a patent. Such action is therefore respectfully solicited.

Rejection Under 35 U.S.C. §112

Claims 109-112 are rejected under 35 U.S.C. §112, ¶1, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner stated that this is a "new matter" rejection.

The Examiner stated with regard to the subject rejection that Applicants point to page 32, lines 1-33 and page 12, lines 4-14 for support for the invention recited in claims 109-122. The Examiner then further stated that the cited portions of the specification are not persuasive, insofar as enablement is concerned, in that the passage at page 32, lines 4-14 provides for a *specific coupling procedure at the C-4 carbon of the sphingosine moiety of the ceramide to the ε-aminolysyl group of a protein* (the procedure involves ozonolysis, production of a functional aldehyde group and coupling to an ε-aminolysyl group on a protein by reductive amination). The Examiner additionally stated that the passage at page 12, lines 4-14, in combination with the passage at page 32, lines 1-33, does not support a broad coupling of the ceramide backbone of the ganglioside at the C-4 carbon of the sphingosine moiety by any generic means to the immunogenic protein based carrier. The Examiner stated that the text at pages 12 and 32 does not support, by way of written description, that Applicants had at

the time of filing contemplated coupling by any means other than the C-4 carbon of the sphingosine moiety of the ceramide to the ε-aminolysyl group of proteins. The Examiner stated that Applicants' amendment (i.e., the introduction of new claims 109-122) provides a new genus of coupling procedure at the C-4 carbon of the sphingosine moiety of the ceramide to the immunogenic protein based carrier by any means and any chemistry. The Examiner stated that this genus concept is not supported by the specific written description. The Examiner further stated that the linkage by means of the C-4 carbon is tied to coupling through the ε-aminolysyl group of a protein and that Applicants were clearly not in possession of this new genus.

The Examiner went on to state that the court's decision in In re East and Harmon (CCPA) 181 USPQ 716 (May 9, 1994) is instructive. The Examiner noted that in the case in question the claims of a reissue application were found to be drawn to new matter since they broadly recited a genus of "carrier particles" not disclosed in the original patent, which disclosed only the subgenus of "magnetic carrier particles" and species of "iron, ferrites, nickel, and cobalt" carrier particles. The Examiner stated that the genus now claimed in the present application is not supported by the specific species described in the specification, as set forth *supra*. The Examiner stated that the genus of types of linkages to the immunogenic protein-based carrier is not supported by the specification as originally filed.

In response to the above ground for rejection, Applicants submit that claim 109, i.e., the sole independent claim present in this application, from which all of the remaining claims depend, has now

been amended to recite that the immunogenic protein-based carrier comprises an ε-aminolysyl group, and further that:

> in the conjugate the ganglioside derivative is covalently bound to the immunogenic protein based carrier by a stable amine bond between the C-4 carbon of the altered sphingosine base of the altered ceramide portion of the ganglioside derivative and the nitrogen of the ε-aminolysyl group of the immunogenic protein-based carrier. (emphasis supplied).

In support of the above-described amendments the Examiner's attention is respectfully directed to page 32 of the specification which teaches, at lines 15-20, concerning the conjugation of the ganglioside to the protein, that:

> The ceramide, characteristic for all gangliosides, was cleaved with ozone at the C-4 position of the sphingosine base and a functional aldehyde group was introduced. Coupling to protein was realized by reductive amination to form a stable amine bond between the ganglioside and the ε-aminolysyl groups of proteins.

The specification goes on to teach, moreover, that the above described method of conjugation was used by Applicants with gangliosides other than the GD3 ganglioside. For example, page 65, lines 9-15 of the specification, in describing the preparation of a GM2-Keyhole Limpet Hemocyanin vaccine, teaches that:

> GM2-KLH conjugate was prepared ... as described previously for GD3-KLH conjugate vaccine. Briefly, the conjugation

procedure involved ozone cleavage of the ceramide double bond of GM2, introduction of an aldehyde group, and conjugation of aminolysyl groups of KLH by reductive amination.

The disclosure of the present application thus clearly teaches to one of ordinarty skill in this art the conjugation of a ganglioside derivative to an immunogenic protein-based carrier by a stable amine bond between the C-4 carbon of the altered sphingosine base of the altered ceramide portion of the ganglioside derivative and the ϵ-aminolysyl group of the immunogenic protein-based carrier (e.g., KLH).

As amended, moreover, claim 109 does not recite a coupling of the ceramide backbone of the ganglioside at the C-4 carbon of the sphingosine moiety by any generic means to the immunogenic protein-based carrier. Rather, the claim now recites, as particularly taught in the cited portions of the specification(see above), a specific coupling obtained with a stable amine bond formed between the C-4 carbon of the altered sphingosine base of the altered ceramide potion of the sphingosine derivative and the ε-aminolysyl group on the protein. As noted, the specification clearly conveys to one of ordinary skill in this art the mode of conjugation now recited in Applicants' (amended) claim 109, and thus in claims 110-122 which depend from claim 109.

In light of the amendments to claim 109, therefore, the Examiner is respectfully requested to reconsider and withdraw her rejection under 35 U.S.C. §112, ¶1, of claim 109, as well as remaining claims 110-122, each of which, as noted above, depends from claim 109.

Rejection Under 35 U.S.C. §103(a)

Claims 109-122 are rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over Livingston et al. (Cancer Research, 149:7045-7050, 1989) in view of Ritter et al. (Seminars in Cancer Biology, 2:401-409, 1991), Liane et al. (Journal of Biological Chemistry, 249(14):4460-4466, 1974), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol., 182:32-43, 1990), Kensil et al. (The Journal of Immunology, 146(2):431-437, 1991), Marciani et al.(Vaccine, 9:89-96, 1991) and Uemura et al. (J. Biochem, 79(6):1253-1261, 1976) for the reasons made of record in Paper No. 20, i.e., the USPO Office Action mailed April 11, 2000. These reasons are reiterated below for the sake of clarity.

In the subject Office Action the Examiner stated that Livingston et al. (Cancer Research) teach a composition administered to melanoma patients for stimulating the production of antibodies directed against a carbohydrate epitope on the ganglioside, GM2 (page 7046-7048) and that the composition for treatment is administered at a concentration of 100, 200 or 300 μg with an adjuvant, Bascillus-Calmetter-Geurin (BCG), and a pharmaceutically acceptable vehicle, phosphate buffered saline, (p. 7046, column 1, paragraph 3, and paragraph bridging p. 7046-47). The Examiner further stated that Livingston et al. teach that melanoma recurrence was delayed in patients developing GM2 antibodies after treatment with the composition (page 7048, paragraph 1 and column 2, paragraph 2). The Examiner also stated that Livingston et al. teach that more patients produced IgM antibodies than IgG antibodies to the GM2 (page 7047, paragraph bridging columns 1-2), and that the

gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas (page 7045, column 1, paragraph 2). The Examiner additionally stated that Livingston et al. teach treatment of a melanoma, a cancer which is both epithelial and neuroectodermal in origin, and that Livingston et al. differ [from the invention] by not teaching the conjugation of the GM2 or other gangliosides by means of a carbon on the ceramide moiety with aminolysyl groups on Keyhole Limpet Hemocyanin (KLH) in a composition and also by not using the composition for treatment.

The Examiner also stated that Ritter et al. (1991) teach that IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides, resulting in the T cell help necessary for the response (page 406, paragraph 1). The Examiner further stated that Ritter et al. discloses that the advantage of inducing an IgG antibody response (vs. IgM) against gangliosides is that IgG: a) has a higher affinity, b) is better able to penetrate solid tissues, C) is able to mediate antibody-dependent cell-mediated cytotoxicity, d) and is generally detectable in the serum for longer periods after immunization.

The Examiner additionally stated that Liane et al. (Journal of Biological Chemistry, 249(14):4460-4466, 1974) teach a method of covalent coupling of gangliosides to aminoethyl agarose or amino group bearing glass beads by oxidative ozonolysis of the olefinic bond of the sphingosine moiety (i.e., the instant carbon double bond of ceramide) and coupling of the carboxyl bearing product to the amino group of aminoethyl agarose or the amino group bearing glass beads.

The Examiner next stated that Ritter et al.,(1990) teach that GD3 lactone is more immunogenic than GD3.

The Examiner further stated that Livingston et al. (U.S. Patent No. 5,102,663) teach that gangliosides GM3, GM2, GD3, GD2, GT3 and O-acetyl GD3 are gangliosides that are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin (column 1, lines 22-28).

The Examiner went on to state that Kensil et al., teach that QS-21 (i.e., the instant carbohydrate derivable from the bark of a Quillaja saponaria Molina tree) produced a higher antibody response than conventional aluminum hydroxide (page 433, column 2, paragraph 4 and Figure 3), and that Kensil, et al., also teach that the immune responses obtained with QS-21, reached a plateau at doses between 10-80µg in mice (page 433, column 1, paragraph 3).

The Examiner stated that Marciani et al. teach that the use of QS-21 adjuvant was useful because it did not cause a toxic reaction in cats (page 93, paragraph 1).

The Examiner stated that Uemura et al., (J. Biochem, 79(6);1253-1261, 1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity of the ganglioside derivative with antibodies.

The Examiner then expressed the conclusion that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to modify the composition taught by

Livingston et al. by conjugating the GM-2 to KLH by covalently coupling GM2 to KLH by substituting GM2 for the globoside and KLH for the aminoethyl agarose to produce a GM-2-KLH conjugate by means of the olefinic bond of the sphingosine moiety of the GM2 (i.e., the instant ceramide double bond) and the ε-aminolysyl groups present in the KLH protein using the method of Liane et al., and to add QS-21 as an adjuvant to the GM-2-KLH conjugate for use as a vaccine because the conjugated composition would be expected to enhance the IgG response to the ganglioside, as taught by Ritter et al. (1991), thus providing the advantages taught by Ritter et al. (1991). The Examiner went on to state that adding the QS-21 would be advantageous because it provides for a higher antibody response than the commonly used adjuvant used by Kensil et al., and that QS-21 provides the advantage that it is not toxic to animals as taught by Marciani et al.. The Examiner further opined that it also would have been *prima facie* obvious to use doses of between 10 and 80μg and that to optimize the weight ratio of the components of the composition to provide an optimal response is well within the ordinary skill in the art, as well as to use the composition as modified supra for treatment of melanoma as taught by Livingston et al. (Cancer Research). The Examiner additionally stated that it also would have been prima facie obvious to one of ordinary skill in the art to substitute any one of GM3, GD2, GD3 or O-acetyl GD3 for the GM2 ganglioside in the composition and method as combined supra because they are all prominent cell membrane components of melanomas as taught by Livingston et al.(U.S. Patent No. 5,102,663) and one of ordinary skill in the art would know they would react with the melanoma cells. The Examiner went on to state that it would have also been prima facie obvious to one of ordinary skill in the art at the time the invention was made to substitute the GD3

lactone for the GM2 ganglioside in the composition because GD3 lactone is more immunogenic than GD3, as taught by Ritter et al. (1990) and would be expected to produce an enhanced antibody response as compared to GD3. The Examiner further stated that optimization of the dosage, the route of immunization and the number of sites of immunization to administer the composition is well within the skill of the ordinary artisan. The Examiner stated that one would have reasonably expected the conjugation procedure to work as substituted because conjugation through the ε-aminolysyl groups of carrier proteins for enhanced immunogenicity is routine in the art. The Examiner further noted that Uemura et al. (J. Biochem, 79(6);1253-1261, 1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies.

The combination of references cited to reject applicants' claims was discussed in detail in Applicants' "Amendment In Response to January 17, 2001 Communication", filed July 23, 2001. In particular, Applicants set forth therein the features of the invention which, in their view, distinguish claims 121-134 [sic now renumbered as claims 109-122] over the cited references. The comments and arguments in Applicants' July 23, 2001 submission are specifically incorporated by reference into the present response and thus will not be repeated here.

In response to Applicants' arguments in the July 23, 2001 Amendment, the Examiner characterized Applicants' position in her remarks set forth in the Final Office Action dated October 5, 2001, stating in this regard that Applicants argue that the combination fails because Ritter (1991) fails to teach the means of chemical

coupling. The Examiner stated that the means of chemical coupling of gangliosides was known to the art, and that the key teaching of Ritter (1991) is that one would expect a better response by means of coupling the ganglioside to the KLH. The Examiner stated that Ritter (1991) provides motivation to make conjugates of gangliosides and KLH with the expectation of better immunogenicity. The Examiner stated that Applicants misrepresented the Examiner's reliance on Ritter (1990) in that Ritter (1990) was cited solely for the teaching that GD3 lactone is more immunogenic than GD3 and to support the substitution of the lactone for the GD3 as combined and was never cited to represent linkage through ceramide. The Examiner stated that Applicants' misdirection of the rejection is not understood. The Examiner stated that Applicants apparently argue teachings not relied upon by the Examiner. The Examiner stated that the modification is of the composition of Livingston et al., not of Ritter (1991) or Ritter (1990). The Examiner stated that Applicants also argue that Liane et al. does not supply what is missing from the primary reference. The Examiner stated that this is not persuasive, as Liane et al. teaches conjugation by means of the C-4 carbon. The Examiner stated that Helline et al. (Exhibit A to Applicants' prior response) opined that Liane et al. is of limited use to the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. The Examiner stated that in contrast to Helline's statement, Liane et al. does not require deacetylation after conjugation with aminoethyl-sepharose. The Examiner stated that Helline et al. therefore misrepresents the complete teachings of Liane et al. The Examiner stated that figure 1, page 4461, provides carbodiimide linkage under standard acidic, not basic conditions. The Examiner

stated that the deacetylation occurs before the linkage step and the protein is not present in basic conditions when substituted for the sepharose. The Examiner stated that the teachings of Liane et al. (scheme 1) do not require decetylation of the sepharose conjugate under basic conditions as alleged by Applicants. The Examiner stated that, moreover, the use of carbodiimide under the conditions of Liane et al. have long been use for coupling of peptides to carrier proteins and will not degrade the protein.

Responding, therefore, to the grounds for rejection as set forth above, Applicants submit that independent claim 109 has now been amended in its description of the mode of conjugation between the ganglioside derivative and the immunogenic protein-based carrier to recite means for covalently binding the two moieties which is nowhere taught or even suggested in any of the references cited to reject the claims of the present application, taken alone or in any combination.

That is, as now amended, claim 109 (i.e., the sole independent claim in the application), is directed to a composition which comprises:

a) a conjugate of i) a ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising an altered sphingosine base, to ii) an immunogenic protein-based carrier comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier,

the relative amount of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject,

wherein the ganglioside derivative is a derivative of a ganglioside selected from the group consisting of GM2, GM3, GD2, GD3, GD3 lactone, O-acetyl GD3 and GT3 and wherein the immunogenic protein based carrier is derived from a protein selected from the group consisting of malaria T-cell epitope, an outer membrane protein of Neisseria meningitidis, cationized bovine serum albumin, Keyhole Limpet Hemocyanin, polylysine and human serum albumin,

wherein in the conjugate the ganglioside derivative is covalently bound to the immunogenic protein-based carrier by a stable amine bond between the C-4 carbon of the altered sphingosine base of the altered ceramide portion of the ganglioside derivative and the nitrogen of the ε-aminolysyl group of the immunogenic protein-based carrier (emphasis supplied).

As pointed out by the Examiner in Paper No. 20, i.e., the Office Action mailed April 11, 2000, Liane, et al. (Journal of Biological Chemistry, 249(14);4460-4466 (1974)) teach a method for covalent coupling of gangliosides to aminoethyl agarose or amino group bearing glass beads by oxidative ozonolysis of the olefinic bond of the sphingosine moiety (i.e., the instant carbon double bond of the ceramide) and coupling of the carboxyl bearing product to the amino group of aminoethyl agarose to the amino group bearing glass beads. Thus, as illustrated for example in Fig. 1 of Liane et al., the

coupling is through a carbon which forms part of a C=O group.

In contrast, and as noted above, claim 109 as amended now specifically states that the ganglioside derivative of the composition of the present invention is covalently bound to the immunogenic protein-based carrier by a stable amine bond between the C-4 carbon of the altered sphingosine base of the altered ceramide portion of the ganglioside derivative and the nitrogen of the ε-aminolysyl group of the carrier, which arrangement is nowhere taught or even suggested in the subject Liane, et al. reference.

As the presently claimed mode of linkage is thus neither taught nor suggested by the Liane et al. reference, nor any of the other references cited in combination with Liane et al. to reject the claims of the present application, Applicants respectfully submit that the invention as now recited in (amended) claim 109, as well as the claims which depend therefrom, is not obvious to one of ordinary skill in the art. Thus, the rejection of claims 109-122 under 35 U.S.C.§103(a) should be reconsidered and withdrawn.

Supplemental Information Disclosure Statement

In compliance with their duty of disclosure under 37 C.F.R. §1.56, Applicants direct the Examiner's attention to the following references, which are listed on accompanying Form PTO-**1449 (Exhibit 2)**, copies of which are attached hereto as **Exhibits 3-4.**

Wiegand, et al., U.S. Patent No. 5,599,914, issued February 4, 1997, filed November 24, 1989 **(Exhibit 3)**.

Fiume, L. et al., Critical Reviews In Therapeutic Drug Carrier Systems, 4(4):265-284, 1998 **(Exhibit 4)**.

These references were cited in Office Actions dated August 27, 2002 issued in two related applications (i.e., Serial No. 08/477,097 and Serial No. 08/475,784, respectively) to the present application. Applicants maintain that the subject references neither disclose nor suggest the invention claimed in the present application, whether viewed alone or in combination with any other(s) of the cited references.

In accordance with 35 C.F.R.§1.97(e)(2) the undersigned certifies that no item of the information contained in this Information Disclosure Statement was cited in a Communication from a foreign Patent Office in a counterpart foreign application, and to the knowledge of the undersigned after making reasonable inquiry, no item of information contained in this Information Disclosure Statement was known to any individual designated in 36 C.F.R. §1.56 (C) more than three months prior to the filing of this Information Disclosure Statement.

No fee under 37 C.F.R.§1.97 (d) is believed to be due for the filing of this Information Disclosure Statement since the filing of the present response under 37 C.F.R. §1.129(a) serves to withdraw the finality of the October 5, 2001 Office Action. Thus submission of the subject references is being made under §1.97(c). If any fee is due, however, authorization is hereby given to charge the required amount to Deposit Account No. 03-3125.

Summary

For all of the reasons set forth hereinabove, Applicants respectfully request that the Examiner reconsider and withdraw the various grounds of rejection, and earnestly solicit the allowance of claims 109-122 presently pending in this application.

If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicants' attorneys invite the Examiner to telephone at the number below.

A $720.00 fee for a four month extension of time, together with a fee of $370.00 for filing this submission under 37 C.F.R. §1.129(a), for a total fee of $1090.00 is deemed to be necessary in connection with this filing. A check in the amount of $1090.00 is therefore enclosed. Should any additional fee(s) be due, authorization is hereby provided to charge the amount due to Deposit Account No. 03-3125.

Respectfully submitted,

Mark A. Farley

John P. White
Registration No. 28,678
Mark A. Farley
Registration No. 33,170
Attorneys for Applicants
Cooper & Dunham, LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Mark A. Farley 10/2/02
John P. White Date
Reg. No. 28,678
Mark A. Farley
Reg. No. 33,170

Exhibit 1

Amended Claim

--109. (amended) A composition which comprises:

a) a conjugate of i) a ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising [a] an altered sphingosine base, to ii) an immunogenic protein-based carrier comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier,

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject,

wherein the ganglioside derivative is a derivative of a ganglioside selected from the group consisting of GM2, GM3, GD2, GD3, GD3 lactone, O-acetyl GD3 and GT3; and wherein the immunogenic protein-based carrier is derived from a protein selected from the group consisting of malaria T-cell epitope, an outer membrane protein of Neisseria Meningitidis, cationized bovine serum albumin, Keyhole Limpet Hemocyanin, polylysine and human serum albumin;

wherein in the conjugate the ganglioside derivative is

[conjugated] covalently bound to the immunogenic protein-based carrier by a stable amine bond between the [through a] C-4 carbon of the altered sphingosine base of the altered ceramide portion of the ganglioside derivative and the nitrogen of the ε-aminolysyl group of the immunogenic protein-based carrier.--

Form PTO-1449 U.S. Department of Commerce Patent and Trademark Office	Atty. Docket No. 1747/43016-D	Serial No. 08/477,147
INFORMATION DISCLOSURE CITATION (Use several sheets if necessary)	Applicants Philip O. Livingston et al.	
	Filing Date 6-7-95	Group ~~1645~~ 1642

U.S. PATENT DOCUMENTS

Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date if Appropriate
AH		5 5 9 9 9 1 4	11/24/89	Wiegand et al.	Exhibit	3	

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

AH		Fiume, L. et al., Critical Reviews in Therapeutic Drug Carrier Systems, 4(4): 265-284, 1988 (Exhibit 4).

EXAMINER	Anne L. Allen	DATE CONSIDERED	12/30/02

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609: Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

US005599914A

United States Patent [19]

Wiegand et al.

[11] Patent Number: 5,599,914

[45] Date of Patent: Feb. 4, 1997

[54] **GLYCOSPHINGOLIPIDS WITH A GROUP CAPABLE OF COUPLING IN THE SPHINGOID PORTION, THE PREPARATION AND USE THEREOF**

[75] Inventors: **Herbert Wiegand; Silke Bosslet**, both of Marburg, Germany

[73] Assignee: **Behringwerke Aktiengesellschaft**, Marburg, Germany

[21] Appl. No.: **440,798**

[22] Filed: **Nov. 24, 1989**

[30] **Foreign Application Priority Data**

Nov. 27, 1988 [DE] Germany 38 40 044.8

[51] **Int. Cl.**[6] **C07G 3/00**; C07G 37/00; C07H 5/04

[52] **U.S. Cl.** **536/4.1**; 536/18.5; 536/18.7; 536/53; 536/55.1; 536/55.3; 530/395

[58] **Field of Search** 536/53, 4.1, 55.1, 536/18.7, 55.3, 18.5; 514/25; 530/395

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,757,003	7/1988	Matsumoto et al.	536/53
4,849,413	7/1989	Della Valle et al.	536/53
4,918,170	4/1990	Hasegawa et al.	536/1.1
4,980,462	12/1990	Karlsson et al.	536/53

FOREIGN PATENT DOCUMENTS

0155625	9/1985	European Pat. Off.	536/53
3837623.7	of 1988	Germany .	

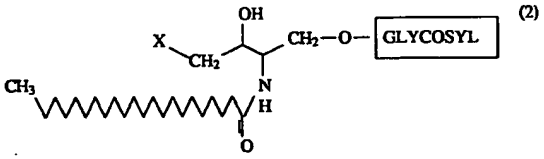
OTHER PUBLICATIONS

H. Wiegandt and G. Beschang, Z. Naturforschung 206 (1965), pp. 164–166.
H. Wiegandt, Ang. Chem. Intl. Ed. 7 (1968), pp. 87–96.
Pappas et al., Tetrahedron Letters, 36 (1966) pp. 4273–4277.
Wiegandt and Ziegler, Hoppe-Seyler's Physiol. Chem. 355 (1974), pp. 11–18.
Itasaka and Hori, J. Biochem. 85 (1979), pp. 1469–1481.
Carlsson et al, J. Biochem. 173 (1978), pp. 723–737.
Roy et al; J. Carbohydrate Chemistry 6(1):161–165 (1987).
Schwarzmann et al; Biochemistry 22:5041–5048 (1983).
Furst et al; Biol. Chem., Hoppe-Seyler's 369(5):317–328 May 1988.
Laine et al, J. Biol. Chem. 249(14):4460–6 (1974).

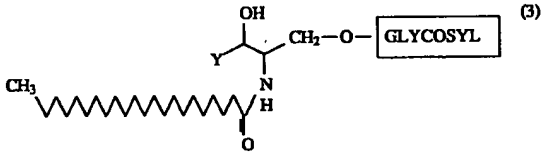
Primary Examiner—John W. Rollins
Attorney, Agent, or Firm—Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

[57] **ABSTRACT**

The invention relates to the chemical modification of the sphingoid portions of glycosphingolipids. It has been possible by a series of reactions to introduce an amino group in the position of the carbon double-bond in the sphingoid portion after elimination of the long-chain aldehyde. Glycosphingolipids of the formula (2) and (3), where X and Y denote a group capable of coupling, are suitable for coupling to other molecules, preferably proteins. X preferably represents NH_2, and Y preferably represents



(2)



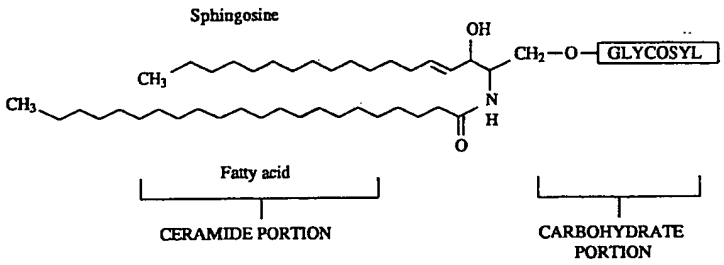
(3)

8 Claims, No Drawings

GLYCOSPHINGOLIPIDS WITH A GROUP CAPABLE OF COUPLING IN THE SPHINGOID PORTION, THE PREPARATION AND USE THEREOF

The invention relates to the chemical modification of the sphingoid portions of glycosphingolipids. It has been possible by a series of reactions to introduce an amino group in the position of the carbon double-bond in the sphingoid portion after elimination of the long-chain aldehyde.

Glycosphingolipids (formula I) are plasma membrane lipids which are composed of a hydrophilic carbohydrate portion and of a hydrophobic ceramide portion. The ceramide portion is composed of sphingosine, a long-chain amino alcohol and a fatty acid bonded as amide.



(I)

Glycosphingolipids are anchored with this double-tailed hydrophobic portion in the outer plasma membrane in such a way that their oligosaccharide chains project into the extracellular space.

Despite intensive research work, the biological function of glycosphingolipids is not yet accurately known; however, they appear to play a part in the regulation of cell growth and differentiation. Findings which show that, in particular, glycosphingolipids containing sialic acid, gangliosides, occur in a relatively large amount on some tumors of neuroectodermal origin, whereas they are expressed in smaller amounts on normal tissue, have attracted interest to them as tumor-associated antigens for tumor diagnosis and tumor therapy.

The carbohydrate portion is of particular interest in this connection. It has already been shown (German Patent Application P 38 37 623.7) that appropriate sialyl-sugars isolated from a source such as cow colostrum and coupled to a carrier protein are able to imitate epitopes of gangliosides: monoclonal antiganglioside antibodies react with these neoglycoproteins.

In order to be able to establish a more universal targeted coupling which can be applied to all glycosphingolipids, chemical modification of the sphingoid portion of glycosphingolipids is necessary for further work in this area. This relates to the preparation of synthetic glycosphingolipid vaccines. In addition, the introduction of a group capable of coupling is also relevant for problems in basic research, for example glycosphingolipids coupled to reporter enzymes can be employed in histochemical investigations, for example for characterizing mammalian lectins as receptors for glycosphingolipids.

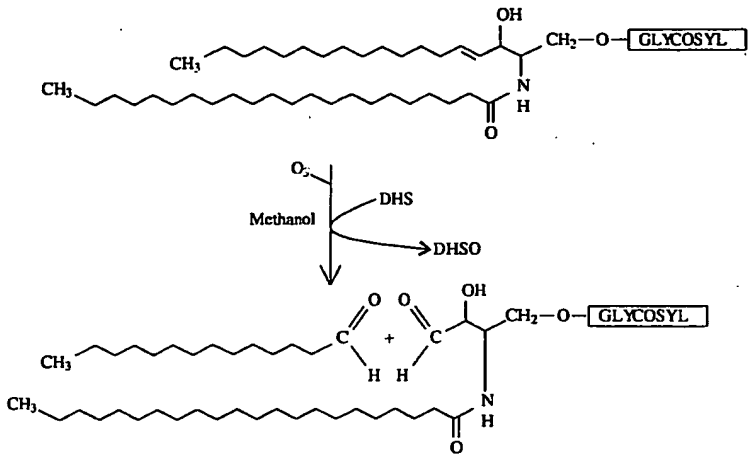
The invention shows that an amino group can, by the reactions described below, be introduced as functional group into the sphingoid portion of glycosphingolipids after elimination of a long-chain aldehyde. During this the glycosidic bonding of the carbohydrate portion to the ceramide portion remains unchanged.

The introduced amino group makes a number of further reactions possible, for example coupling to heterobifunctional reagents for the synthesis of glycosphingolipid conjugates and the use thereof as synthetic vaccines in the therapy of tumors of neuroectodermal origin.

Besides the introduction of an amino group, there is also the possibility of reacting the intermediate (which carries an aldehyde group) directly with other molecules carrying groups capable of coupling, for example amino groups of proteins, coupling reagents etc. It has to be remembered in the reactions that the intermediate with the aldehyde group is not very stable and the carbohydrate portion is eliminated in an alkaline medium.

The principal reactions for introducing the group capable of coupling at the position of the carbon double-bond in the sphingoid portion are as follows:

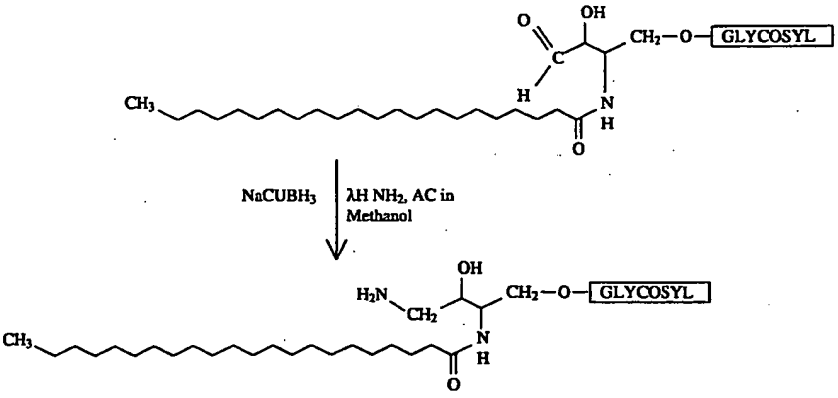
1. The abovementioned double-bond is cleaved by ozone (H. Wiegandt and G. Baschang, Z. Naturforschung 206, (1965), 164–166) and the methoxy hydroperoxide derivatives which are formed in methanol as intermediates (H. Wiegandt, Ang. Chem. Intl. Ed. 7, (1968) 87–96) are reduced to the aldehyde by addition of dimethyl sulfide (Pappas et al., Tetrahedron Letters, 36 (1966), 4273–4278).



The resulting compound is stable only in neutral and acidic media; the carbohydrate portion is eliminated in an alkaline medium.

2. After removal of the long-chain aldehyde by extraction by shaking in hexane, subsequently the ozonolysis product is reductively aminated in methanol with the addition of 1M ammonium acetate and sodium cyanoborohydride (Wiegandt and Ziegler, Hoppe-Seyler's Physiol. Chem. 355, (1974), 11–18).

Accordingly, the invention relates to glycosphingolipids with a group capable of coupling in the position of the carbon double-bond, there being elimination of a long-chain aldehyde while the molecule remains otherwise intact and, in particular, the glycosidic bonding of the sugar portion is retained, and the group capable of coupling preferably being an amino group or an aldehyde group, and to processes for the preparation thereof and to the use thereof for coupling to suitable reactants and as ingredient of pharmaceuticals.



All the reaction steps take place with a very high yield and can be followed by thin-layer chromatography: the mobile phase is chloroform/methanol/water (65:25:4). The reaction products are detected on the thin-layer chromatography plates with iodine vapor or ninhydrin or fluoram and orcinol spray reagent.

The reductively aminated ozonolysis product is ninhydrin- and fluoram-positive on the thin-layer chromatography plate and can be reacted completely with reagents specific for amino groups, such as fluorodinitrobenzene (Sanger's reagent), (Itasaka and Hori, J. Biochem. 85, (1979), 1469–1481) or SPDP (N-succinimidyl 3-(2-pyridyldithio-)propionate) (Carlsson et al., Biochem. J. 173, (1978), 723–737).

The invention is furthermore disclosed in the example and the patent claims.

EXAMPLE A

1. Ozonolysis

In a typical reaction mixture, 6 mg of cerebroside (galactosylceramide) were dissolved in 5 ml of methanol and the mixture was ozonolysed (1 bubble/sec) at room temperature until unconsumed ozone was indicated by the violet color of the KI/starch detection solution.

2. Reduction to the aldehyde

The mixture was subsequently gassed with nitrogen, 30 µl of dimethyl sulfide were added, and the mixture was left to stand overnight. After the solution had been concentrated in a rotary evaporator without heating, the long-chain aldehyde liberated in the ozonolysis was removed by extraction by shaking in hexane (3×2 ml).

3. Reductive amination of the ozonolysis product

The residue from the extraction was dissolved in 4 ml of 1M ammonium acetate in methanol, 15 mg of sodium cyanoborohydride were added, and the mixture was boiled under reflux at 80° C. for 4–5 hours.

The reductively aminated ozonolysis product was subsequently desalted and purified by reversed phase (RP18) chromatography and small silica gel coles.

4. Reaction of the reductively aminated ozonolysis product with fluorodinitrobenzene

An aliquot of the reductively aminated ozonolysis product was dissolved in 500 µl of methanol, and 4 drops of tricthylamine and 20 µl of 5% fluorodinitrobenzene in ethanol were added to the mixture. The reaction was carried out in 1–2 hours at room temperature, shaking occasionally.

The resulting dinitrophenyl derivative was immediately identifiable because of its yellow color on the thin-layer chromatography plate (HPTLC plate, silica gel **60** (Merck, Darmstadt); mobile phase: chloroform/methanol/water, 65:25:4).

EXAMPLE B

Coupling of reductively aminated ozonolysis products of the gangliosides GM3, GD3, GM2 and GM1 to human serum albumin (HSA) by means of the heterobifunctional coupling reagent N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP); synthesis of conjugates with a derivatization level of 16–18 ganglioside derivatives per HSA molecule

1. Preparation of the reductively aminated ozonolysis products of the gangliosides GM3, GD3, GM2 and GM1 The preparation was carried out as described for cerebroside under 1.–3. in Example A. Mass spectrum analyses of the GM1 and GM3 derivatives confirmed the expected structure.

Subsequent reaction steps:

2. reaction of the reductively aminated ozonolysis products with SPDP

3. reaction of HSA with SPDP

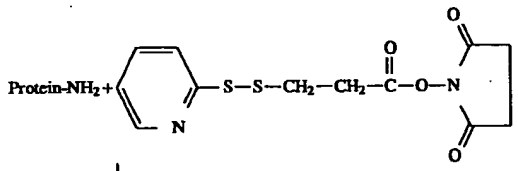
4. reduction of the HSA-SPDP derivative

5. coupling of the ganglioside derivative to the protein derivative and the corresponding detection methods substantially correspond to the method of J. Carlsson et al. (1987) Biochem. J. 173, 723–737 and as proposed in Patent Application DE P 38 37 623.7. Steps 2. and 3. were carried out in 0.1M sodium phosphate buffer, pH 7.5, with a 3- to 5-fold molar excess (based on free epsilon-aminolysyl groups in 3.) of SPDP. The removal of the protein-SPDP derivative from 3. was carried out on a Sephadex G-25 column which was eluted with the buffer for the subsequent reactions (0.1M sodium phosphate buffer, pH 6, 5 mM EDTA). The ganglioside derivatives reacted with SPDP were purified by reversed phase (RP18) chromatography. The individual intermediates were identified by thin-layer chromatography on the basis of the change in the migration behavior on silica gel G-60 plates in the mobile phases chloroform/methanol/0.2% aqueous calcium chloride (65:25:4) or (50:40:10).

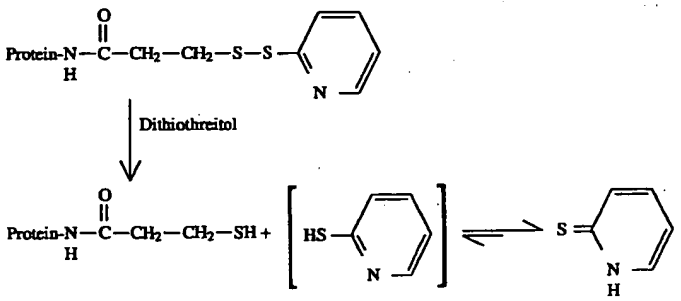
Step 4. was carried out as follows:

The disulfide bridges newly introduced in the HSA-SPDP derivative by the derivatization were reduced, with the addition of 25 mM dithiothrietol, in 0.1M sodium phosphate buffer, pH 6, 5 mM EDTA, with the elimination of 2-thiopyridone. The native disulfide bridges in the protein are not reduced under these reaction conditions. The reaction was carried out at room temperature, and the reaction time was 1–2 hours.

(3)



(4)



The reduced HSA-SPDP derivative was removed on a Sephadex G-25 column with 0.1M sodium phosphate buffer, pH 6, 5 mM EDTA as eluting buffer.

It was possible by reacting SPDP with excess HSA to prepare specific HSA derivatives with a desired derivatization level.

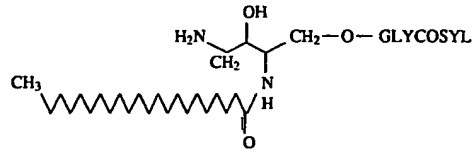
The ganglioside derivatives were reacted with HSA which was derivatized with 16–18 SPDP molecules. Reaction was complete; the derivatization level of the coupling product was 16–18 ganglioside derivatives (each of the gangliosides GM3, GD3, GM2 and GM1) per HSA molecule.

The specific procedure for the coupling (reaction step 5.) was as follows:

Reduced HSA-SPDP derivative was immediately reacted with the ganglioside-SPDP derivative. The ganglioside-SPDP derivative was employed in a 1–5-fold molar excess based on epsilon-aminolysyl groups in the protein, and the reaction time was 24–48 hours at room temperature.

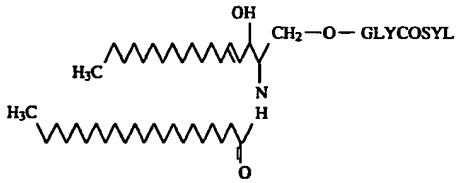
We claim:

1. A process for the preparation of a compound of the formula (III)



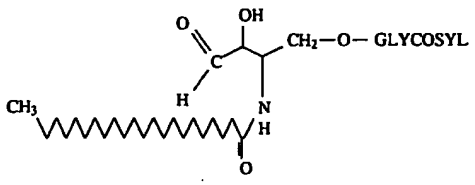
(III)

in which glycosyl is the carbohydrate portion of GM3, GD3, GM2 or GM1, which comprises oxidizing a glycosphingolipid of the formula (I)



(I)

with ozone, and subsequently reducing the reaction product of said oxidizing to a compound of the formula (II),



(II)

and further comprising the reductive amination of said compound of formula (II) wherein glycosyl is as defined above, with an alkali metal cyanoborohydride.

2. The process as claimed in claim 1 which further comprises coupling a compound of formula (III) through the amino group—NH_2 resulting from said reductive amination to another moiety.

3. The method as claimed in claim 2 wherein the other moiety is selected from a heterobifunctional reagent and a protein.

4. The method as claimed in claim 2 wherein the other moiety is a heterobifunctional reagent.

5. The method as claimed in claim 2 wherein the other moiety is a protein.

6. A pharmaceutical which contains a pharmaceutically effective amount of a compound produced by the process of claim 1 coupled to a heterobifunctional reagent.

7. A pharmaceutical which contains a pharmaceutically effective amount of a compound produced by the process of claim 1 coupled to a protein.

8. The process as claimed in claim 1 wherein the reaction product is reduced by addition of dimethyl sulfide.

* * * * *

CRITICAL REVIEWS in THERAPEUTIC DRUG CARRIER SYSTEMS

Volume 4 Issue 4

1988

0743-4 63

TARGETING OF ANTIVIRAL DRUGS BOUND TO PROTEIN CARRIERS

Authors: **Luigi Fiume**
C rrado Busi
Alessandro Mattioli
Giulia Spinosa
Dipartimento di Patologia Sperimentale
University of Bologna
Bologna, Italy

Referee: Giuseppe Barbanti-Brodano
Institute of Microbiology
School of Medicine
University of Ferrara
Ferrara, Italy

I. INTRODUCTION

In the therapy of some diseases, the side effects of drugs would be reduced by introducing them selectively into those cells where their action is required. One approach to this goal consists of coupling the pharmacological agent to a macromolecular vector which is specifically taken up by the target cells. The properties required by the conjugate in order to increase the chemotherapeutic index of the drug can be summarized as follows.[1] The carrier should be (1) transferred into the lysosomal compartment, (2) degradable, (3) neither toxic nor immunogenic, and (4) permeable to the anatomical barriers separating the administration site and the target. The drug should be resistant to lysosomal enzymes and acidic pH. The drug-carrier conjugate should be (1) stable in the bloodstream and extracellular spaces, (2) neither toxic nor immunogenic, and (3) sensitive to the lysosomal enzymes or pH so that the drug can be released in an active form.

The purpose of this review is to describe and evaluate the experiments in which this chemotherapeutic strategy was applied to antiviral drugs. Antiviral agents were conjugated with albumin, galactosyl-terminating glycoproteins, antibodies against an Epstein-Barr virus (EBV) antigen, and horseradish peroxidase. The corresponding conjugates were directed to liver macrophages, hepatocytes, EBV-infected cells, and trigeminal ganglion neurons. The majority of experiments were performed with hepatocyte-addressed conjugates prepared with the aim of increasing the chemotherapeutic index of antiviral drugs in the treatment of chronic hepatitis B virus (HBV) infection. Carrier-mediated delivery of drugs is an appropriate chemotherapeutic approach to the treatment of this disease. In fact: (1) the bulk of HBV grows in hepatocytes, although in some patients virus DNA synthesis also has been observed in nonhepatic cells; (2) hepatocytes specifically internalize galactosyl-terminating glycoproteins which can therefore work as selective hepatotropic vectors of drugs; (3) conjugates can freely come into contact with the surface of hepatocytes since liver sinusoids are not a barrier for proteins.

II. ALBUMIN CONJUGATES

Targeting of antiviral drugs was attempted for the first time in mouse Kupffer cells.[2] The idea of concentrating antiviral agents in these cells arose from the finding that an amanitin-albumin conjugate, prepared with the aim of obtaining a serum neutralizing this toxin,[3] selectively damaged (in vitro[4,5] and in vivo[6,7]) cells displaying a high uptake of denatured albumin such as macrophages and proximal convoluted tubule cells of kidney. In these cells, the amanitin-albumin conjugate produced the ultrastructural changes characteristic of aman-

itin poisoning.[8] The conjugate probably exerted its damaging effect by releasing the toxin after penetration into the cells and cleavage of drug-protein bond(s) in lysosomes.[6] The possibility was considered,[2,5,9] therefore, that other drugs covalently linked to albumin might be concentrated and released in an active form in cells with a high uptake of this protein. If the drugs were selective inhibitors of DNA synthesis, the conjugates would hinder the replication of DNA viruses in macrophages without damaging these cells, which do not divide,[10] and without affecting proliferating cells which do not take up albumin. Two inhibitors of DNA synthesis — 5-fluorodeoxyuridine (FUDR) and cytosine arabinoside (ara-C) — were conjugated to rabbit serum albumin (RSA) by means of 1-ethyl-3-(dimethyl-aminopropyl)carbodiimide (ECDI).[2,11] In in vitro experiments, it was found that FUDR-RSA and ara-C-RSA inhibited the replication of Ectromelia and Vaccinia viruses growing in L-929 cells or mouse peritoneal macrophages.[2] Thymidine and deoxycytidine, which are known to remove the effects of FUDR and ara-C, respectively,[12] counteracted the action of FUDR-RSA and ara-C-RSA, indicating that the activity of the conjugates was due to the FUDR and ara-C moieties. The drugs were released free into the cells; in fact, the bonds linking them to albumin involve their primary hydroxyl group,[13] which must be phosphorylated for the drugs to be able to block DNA synthesis.[12] Mice infected with Ectromelia virus were chosen as an experimental model to study the antiviral activity of these conjugates in vivo.[2] Ectromelia virus, when injected intravenously into mice, is ingested mainly by the Kupffer cells where it replicates. After about 12 hr, it also infects neighboring hepatocytes which in turn infect more hepatic cells after each cycle of growth.[14,15] Coupled FUDR and ara-C injected into mice at the same time as Ectromelia virus reduced — 48 hr after infection — the virus yield in liver by 1 to 2 logs, wheras the free drugs—administered in doses 15 to 20 times higher — were completely ineffective. ara-C-RSA significantly increased the mean survival time of infected mice and the number of survivors in contrast to free ara-C, which was inactive. The conjugates were active only if administered during the period in which the replication of virus is restricted to Kupffer cells.

In conclusion, these results demonstrated that after coupling to albumin, FUDR and ara-C were able to inhibit Ectromelia virus growth in liver macrophages and gave experimental support to the possibility of obtaining a targeting of antiviral drugs by conjugation with protein carriers.

III. GLYCOPROTEIN CONJUGATES

The results obtained with albumin conjugates encouraged[2,16] the attempt of concentrating antiviral agents into hepatocytes by conjugation with galactosyl-terminating glycoproteins. These proteins specifically penetrate, by a receptor-mediated endocytosis, parenchymal liver cells where they are digested in lysosomes.[17,18] The aim of the research was to obtain conjugates potentially useful in the treatment of chronic HBV infection (see Section I).

A. Asialofetuin Conjugates

In the first experiments, asialofetuin (AF) was chosen as the carrier of these hepatocyte-directed conjugates because of its strong affinity for the liver receptor[17] and because it had already been successfully employed to deliver proteins[19] and liposomes[20] to parenchymal hepatic cells.

1. Conjugate with Trifluorothymidine

Trifluorothymidine (F_3T), a powerful inhibitor of deoxyribovirus growth,[21] was linked to AF.[16] The drug was first coverted to F_3T-glutarate, which was subsequently coupled via its hydroxysuccinimide ester to ϵ-NH_2 groups of lysine residues of AF. The molar ratio F_3T to AF in the conjugate was 8. Coupling with F_3T did not change the capacity of AF to interact with the specific receptors on the surface of hepatocytes. Indeed, the clearance of [^{14}C]-

labeled AF from the blood of mice was inhibited to the same extent by F_3T-AF or by an equal amount of nonconjugated AF. The ability of the conjugate to selectively deliver F_3T to liver cells was studied by determining its effect on DNA synthesis in liver and bone marrow of mice 44 hr after the intravenous (i.v.) administration of Ectromelia virus (2×10^5 pfu/g body weight). At this time after infection, the incorporation of deoxyribonucleosides into hepatic DNA, which in normal mice is poor and takes place mainly in sinusoidal cells,[22] is increased four to six times,[16] and the newly synthesized liver DNA hybridizes with viral DNA.[23] Electron microscopic observations demonstrated that the number and size of viral factories as well as the number of viral particles they contain were several times higher in parenchymal than in sinusoidal cells.[23] Therefore, at this point of infection, the largest amount of deoxyribonucleoside incorporation into liver DNA is presumably because of virus DNA synthesis, most of which occurs in hepatocytes.

As shown in Figure 1 at the three lower doses, F_3T coupled to AF caused an inhibition of DNA synthesis in liver more than three times higher than that produced by the free drug. On the contrary, the percentage of inhibition in bone marrow was similar either when F_3T was administered coupled to AF or as a free drug. These results indicate that, after injection of the conjugate, F_3T was concentrated in liver in a pharmacologically active form. Evidence has shown that the inhibition of DNA synthesis in bone marrow of mice injected with F_3T-AF was due to F_3T released from the conjugate in liver escaping from hepatic cells into the blood as a free drug.[16]

2. Conjugates with 9-β-D-Arabinofuranosyladenine (ara-A) and Its 5'-Monophosphate (ara-AMP)

In subsequent experiments,[24] AF was conjugated with 9-β-D-arabinofuranosyladenine (ara-A) because in the meantime this drug was reported to suppress HBV growth.[25,26] In chronic hepatitis B, ara-A and its 5'-monophosphate (ara-AMP)* are effective in producing inhibition of HBV replication and, in a significant proportion of patients, this effect is long lasting with clinical and biochemical evidence of improvement in liver disease.[25,26,28-41] However, ara-A and ara-AMP also produce dose-dependent side effects, mostly neurological disturbances which often necessitate discontinuation of their administration.[35,42-45]

Two different types of conjugate were prepared. In the first, ara-A glutarate was linked to AF via its hydroxysuccinimide ester. The conjugated material (ara-A-glut-AF) emerged from a Sephadex G-75 column in two peaks (A and B). Peak A contained oligomers of AF, whereas peak B contained the monomeric form of AF. The molar ratio of ara-A to AF in both conjugates (A and B) was 8. The second type of conjugate was prepared by coupling ara-AMP to AF by the use of ECDI in an acidic medium (pH about 5). The molar ratio of ara-AMP to AF in 3 preparations of ara-AMP-AF conjugate (I, II, and III) ranged from 3.5 to 4. Table 1 shows that free ara-A and ara-AMP administered to Ectromelia virus-infected mice, during the phase of virus replication in hepatocytes, produced a greater inhibition of DNA synthesis in intestine than in liver. On the contrary, ara-A-glut-AF and ara-AMP-AF inhibited DNA synthesis in liver without producing any significant inhibition in the intestine. A comparable inhibition of DNA synthesis in the liver was obtained with doses of conjugated ara-A and ara-AMP two to four times lower than those of free drugs. These results indicate that targeting of ara-A and ara-AMP was accomplished in liver by using these conjugates. In the case of ara-AMP-AF, the ester bond linking the phosphate to ara-A may be broken down in hepatocyte lysosomes so that it cannot be excluded that the drug released from this conjugate is, at least in part, ara-A.

* Because of very limited solubility, ara-A needs to be administered by continuous i.v. infusion. ara-AMP is at least 400 times more water soluble and is therefore suitable for intramuscular (i.m.) administration. ara-AMP shares the same metabolic pathway as ara-A.[27]

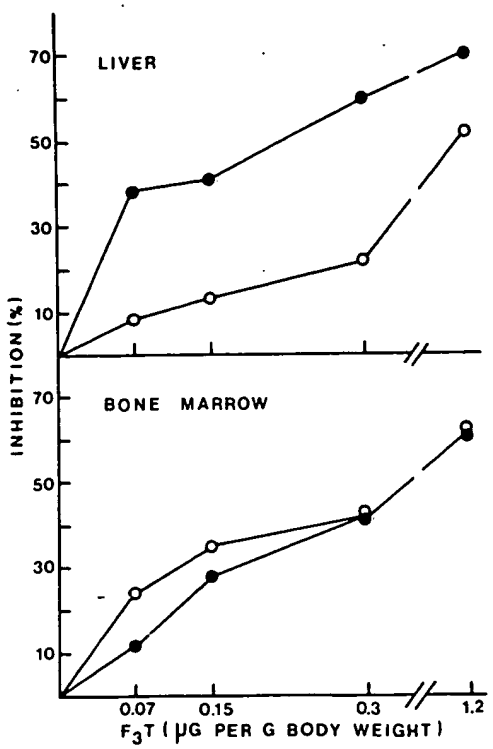


FIGURE 1. Inhibition of DNA synthesis by free F_3T (○) or F_3T coupled to AF (●) in liver and bone marrow of Ectromelia virus-infected mice. For each dose of free and conjugated F_3T, two or three experiments were performed. Results (dpm/mg DNA) were statistically evaluated by means of Student's *t*-test. The difference between deoxy-[5-^{3}H]-cytidine incorporation into liver DNA in mice treated with free F_3T or F_3T-AF was statistically significant for F_3T doses of 0.07, 0.15, and 0.3 μg/g body weight ($p < 0.001$, 0.05, and 0.001 for the 3 doses, respectively). The difference was not statistically significant ($p > 0.05$) for the F_3T dose of 1.2 μg/g body weight. The difference was not statistically significant for all the F_3T doses in bone marrow. (From Fiume, L., Mattioli, A., Balboni, P. G., Tognon, M., Barbanti-Brodano, G., De Vries, J., and Wieland, Th., *FEBS Lett.*, 103, 47, 1979. With permission.)

B. Lactosaminated Albumin Conjugates

A drawback of clinical use of AF conjugates of ara-A and ara-AMP is their immunogenicity. These conjugates are strong inducers of antibodies[46] which can both inactivate the conjugate and produce allergic lesions. This problem may be overcome by using lactosaminated homologous albumin as hepatotropic carrier of the drugs. Some years ago it was suggested[47] that a protein may be made to penetrate into a cell which it does not normally enter by itself by coupling it with a small molecule for which a specific binding site exists on the cell membrane. This hypothesis received support from independent experiments which showed that proteins, after coupling to small sugar molecules, penetrate selectively into cells which possess surface receptors for the coupled carbohydrates.[19,48-50] In this context, it was found that galactosylated proteins, e.g., lactosaminated serum albumin (L-SA), specifically enter into hepatocytes after binding to the receptor for galactosyl-terminating glycoproteins.[48,51] In L-SA, galactosyl residues are attached to albumin by reductive lactosamination

Table 1
INHIBITION OF DNA SYNTHESIS IN LIVER AND INTESTINE OF ECTROMELIA VIRUS-INFECTED MICE AFTER INJECTION OF FREE OR CONJUGATED ara-A AND ara-AMP

Experiment no.	Compound injected	ara-A administered (nmol/g body wt)	Inhibition of DNA synthesis (%)	
			Liver	Intestine
1	ara-A	13.1	42 $p < 0.001$[a]	60 $p < 0.001$
2	ara-A	18.7	58 $p < 0.005$	68 $p < 0.001$
3	ara-AMP	8.6	57 $p < 0.005$	60 $p < 0.001$
4	ara-AMP	17.2	66 $p < 0.001$	77 $p < 0.001$
5	ara-AMP	34.5	77 $p < 0.001$	89 $p < 0.001$
6	ara-A-glut-AF(A)	5.6(33)[b]	38 $p < 0.02$	15 NS
7	ara-A-glut-AF(B)	1.9(11)	26 $p < 0.05$	8 NS
8	ara-A-glut-AF(B)	5.6(33)	43 $p < 0.01$	16 NS
9	ara-AMP-AF(I)	1.8(22)	38 $p < 0.005$	0
10	ara-AMP-AF(II)	1.1(15)	44 $p < 0.001$	0
11	ara-AMP-AF(II)	2.2(30)	59 $p < 0.001$	19 NS
12	ara-AMP-AF(III)	2.8(42)	53 $p < 0.005$	13 NS

[a] Results were statistically evaluated by means of Student's *t*-test. The difference was considered no statistically significant (NS) for $P > 0.05$.

[b] In parentheses is the amount of conjugate injected (μg/g body wt).

From Fiume, L., Mattioli, A., Busi, C., Balboni, P. G., Barbanti-Brodano, G., De Vries, J., Altman, R., and Wieland, Th., *FEBS Lett.*, 116, 185, 1980. With permission.

of ϵ-NH_2 lysine groups in the presence of the cyanoborohydride anion which selectively reduces the imminium salt formed between the aldehydric group of lactose and the lysine residues of the protein.[52,53]

1. Conjugates with ara-AMP

a. Type I Conjugate

The first complexes of lactosaminated albumin with ara-AMP (type I L-SA-ara-AMP) were obtained[23,54] by using L-SAs with 20 to 30 galactosyl residues and following the same procedure employed in the conjugation with AF*.[24] L-SA-ara-AMP conjugates prepared with homo- or heterologous L-SA were taken up selectively by the liver in normal mice: after injection of these conjugates, labeled in the protein moiety by means of [^{3}H] formaldehyde, the values of both acid-insoluble and acid-soluble radioactivities were several times higher in this organ than in spleen, bone marrow, intestine, and brain.[54] In liver cells, ara-AMP (or ara-A) was released free from the conjugate with consequent hepatic targeting of the drug.[23,54] This was demonstrated in Ectromelia virus-infected mice in which, as shown by Figure 2, coupled ara-AMP produced a significant inhibition of DNA synthesis only in liver, whereas the free drug inhibited DNA synthesis in liver, intestine, and bone marrow. In contrast to conjugates prepared with AF or heterologous lactosaminated albumin, the ara-AMP conjugate prepared with homologous L-SA and administered either subcutaneously (s.c.) in complete Freund's adjuvant or infused i.v. was completely devoid of humoral and cellular immunogenicity at least in mice.[46]

A drawback of type I L-SA-ara-AMP conjugates was a rapid loss of solubility which occurred because of polymerization after lyophilization.[55] Polymerization was caused by the

* In the L-SA-ara-AMP conjugates, the molar ratios of lactose to SA and ara-AMP to SA are indicated by subscripts; for example, L_{28}-SA-ara-AMP_{10} is a conjugate with a molar ratio of lactose to SA of 28 and of ara-AMP to SA of 10.

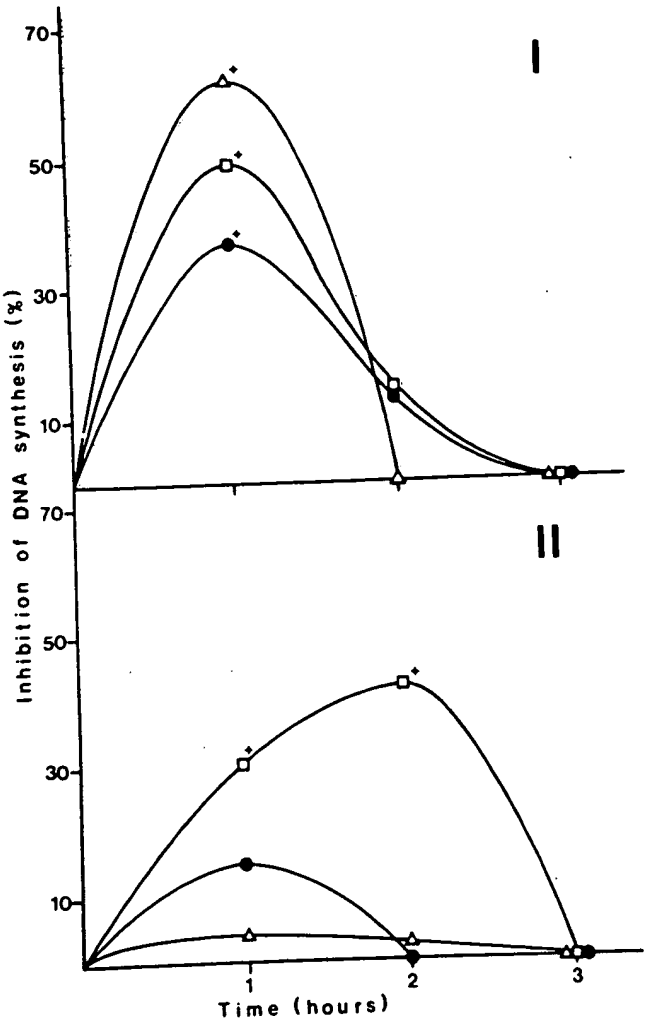


FIGURE 2. Effect of free ara-AMP (I) and L_{28}-H(human)SA-ara-AMP_8 (II) on [methyl-^{3}H] thymidine incorporation into DNA in liver (□), intestine (△), and bone marrow (●) of Ectromelia virus-infected mice. ara-AMP and L_{28}-HSA-ara-AMP_8 were injected i.v. at doses of 7.5 and 35 μg/g body weight, respectively; 35 μg conjugate contains 1.4 μg ara-AMP. Results (dpm/mg DNA) were statistically evaluated by means of Student's *t*-test. (+) means that the difference between [methyl-^{3}H] thymidine incorporation into DNA in treated and control mice was significant ($p<0.05$). (From Fiume, L., Mattioli, A., Busi, C., and Accorsi, C., *Gut*, 25, 1392, 1984. With permission.)

phosphoanhydride bond which links the ara-AMP molecule to L-SA.[55,56] This bond is very reactive and in the presence of an $-NH_2$ group undergoes aminolysis with the formation of a carboamide linkage and release of the phosphate.[57] In the lyophilized conjugate, the bond can react with a lysine ϵ-NH_2 group of another L-SA-ara-AMP molecule, thus causing protein aggregation.

b. Type II Conjugate

By increasing the pH of the coupling medium from 5.5 to 7.5, a new L-SA-ara-AMP conjugate (type II) was obtained which did not polymerize and remained soluble after lyophilization.[55,58] Table 2 shows how the molar ratio of drug to carrier and the solubility of L-SA-ara-AMP conjugates changed by modifying the medium of the coupling reaction.

Table 2
...ECTING THE MOLAR RATIO AND THE SOLUBILITY

Table 2
CONDITIONS OF COUPLING REACTION AFFECTING THE MOLAR RATIO AND THE SOLUBILITY OF L-H (HUMAN) SA-ara-AMP CONJUGATES

Conjugate no.	L-HSA used	Carbodi-imide used	ara-AMP (μmol/mℓ)	Carbodiimide (μmol/mℓ)	pH	Temperature (°C)	Time of incuba-tion (hr)	Conjugates obtained: Molar ratio of ara-AMP to L-HSA	Conjugates obtained: Solubility of conjugate kept at 0 to 4 °C or at room temperature
1	L_{31}-HSA	ECDI[a]	144	261	7.5	25	24	14	Soluble
2	L_{31}-HSA	ECDI	288	522	7.5	25	24	20	Soluble
3	L_{31}-HSA	ECDI	72	130	7.5	25	24	10	Soluble
4	L_{31}-HSA	ECDI	144	130	7.5	25	24	11	Soluble
5	L_{31}-HSA	MCDI[b]	144	261	7.5	25	24	12	Soluble
6	L_{31}-HSA	ECDI	144	261	5.5	25	24	16	Insoluble
7[c]	L_{31}-HSA	ECDI	144	261	6.5	25	24	11	Insoluble
8	L_{31}-HSA	ECDI	144	261	8.5	25	24	10	Soluble
9	L_{31}-HSA	ECDI	144	261	9.5	25	24	5	Soluble
10	L_{31}-HSA	ECDI	144	261	7.5	35	24	9	Soluble
11	L_{31}-HSA	ECDI	144	261	7.5	25	8	11	Soluble
12	L_{20}-HSA	ECDI	144	261	7.5	25	24	11	Soluble
13	L_{51}-HSA	ECDI	144	261	7.5	25	24	12	Soluble

[a] 1-Ethyl-3(dimethylaminopropyl)carbodiimide.
[b] 1-Cycloexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide.
[c] Partially precipitates during conjugation.

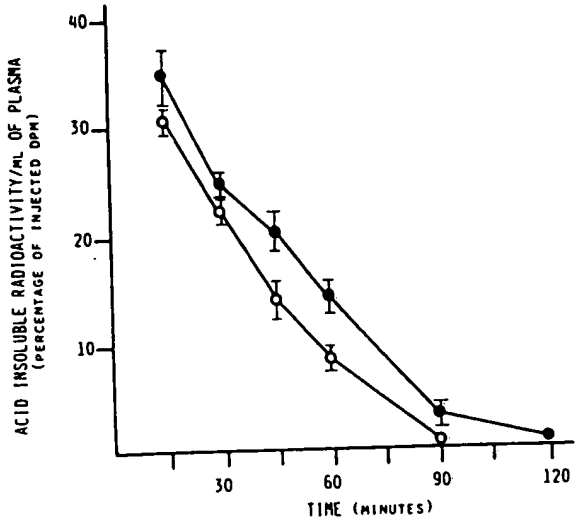


FIGURE 3. Acid-insoluble radioactivities in plasma of mice injected i.v. with conjugates (52 μg/g) labeled in the albumin (●) or the drug moiety (○). Each point represents the mean value of results from four mice. Vertical bars denote S.E. (From Fiume, L., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G., *Biochem. Pharmacol.*, 35, 967, 1986. With permission.)

All the preparations of L-SA-ara-AMP conjugate used in the experiments reported below were obtained in the same medium as the first conjugate of the table. The number of galactosyl residues in the carrier L-SA employed ranged from 29 to 31. The complete procedure for obtaining this conjugate was described by Fiume et al.[58] In several conjugate preparations, the molar ratio of drug to carrier ranged from 12 to 15. Neither the solubility nor the molar ratio was decreased when the conjugate was kept in a lyophilized state at 0 to 4°C for up to 1 year (maximum interval tested). ^{31}P nuclear magnetic resonance (NMR) spectroscopy revealed that in the type II conjugate, ara-AMP is linked to L-SA by two different phosphoamide bonds: one formed with the ϵ-NH_2 group of lysine and the other with one of the imidazole nitrogens of histidine.[56] The two bonds are almost equally represented. They were not formed in type I conjugate presumably because, at pH 5.5, the lysine ϵ-amino groups and the histidine nitrogens are fully or in large part protonated and consequently cannot successfully compete with COO^- groups in the nucleophilic attack of carbodiimide-activated ara-AMP. To test whether only one or both of these linkages allow a rapid intracellular release of the drug, poly(L-lysine) was conjugated with D-galactosyl residues in order to make it a hepatotropic carrier which was subsequently coupled to ara-AMP. In this complex [Gal-poly(L-lysine)-ara-AMP], the only linkage was between the phosphate of the drug and the ϵ-NH_2 groups of lysine residues. Moreover, ara-AMP was coupled to L_{59}-H(human)SA in which all the ϵ-NH_2 groups[59] were substituted by lactose. In this conjugate, the only bond was with histidine, as confirmed by ^{31}P NMR spectroscopy. The two complexes were effective in inhibiting thymidine incorporation in liver of mice with Ectromelia virus hepatitis,[56] thus indicating that both bonds of type II conjugate allow a rapid release of the drug in hepatic cells. As indicated by SDS gel electrophoresis, type II conjugate was composed of the monomer as well as of oligomers of L-SA which were formed as a side reaction during the coupling. They presumably are due to amide formation occurring between carbodiimide-activated carboxylic groups and ϵ-NH_2 of lysine residues.[60] The percentages of monomer, dimer, trimer, tetramer, and heavier oligomers in a typical conjugate preparation were 37, 22, 15, 8, and 18, respectively. These percentages did not change with time,[58] in contrast to the type I conjugate in which a further polymerization takes place after lyophilization (see above).

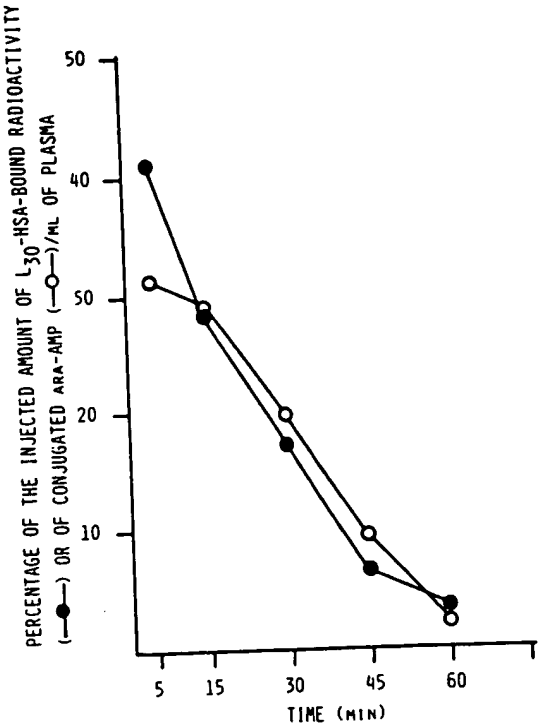


FIGURE 4. L_{30}-HSA-bound radioactivity and coupled ara-AMP (as determined by RIA) in plasma of mice injected i.v. with L_{30}-HSA-ara[^{3}H]AMP_{13} and L_{30}-HSA-ara-AMP_{13}, respectively. The dose of conjugate was 35 μg/g body weight. (From Fiume, L., Balboni, P. G., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G., *Pharm. Acta Helv.*, 61, 342, 1986. With permission.)

Studies on the pharmacokinetics and pharmacology of type II conjugate were performed mainly in mouse. As shown in Figure 3, the levels of radioactivity in the acid-insoluble fraction of mouse plasma after injection of the conjugate labeled in the adenine ring of the drug moiety were only slightly lower than those observed after administration of an equal dose of L_{30}-[^{3}H] HSA-ara-AMP_{14} in which the radioactive label ([^{3}H]-formaldehyde) was linked to protein by a bond which is very strong and not enzymatically cleaved.[61] This indicates that most of the coupled ara-AMP remained linked to L_{30}-HSA.

In the human[27,62] and mouse[63] bloodstream, free ara-AMP is very rapidly dephosphorylated and deaminated to ara-hypoxanthine, a compound several times less active, as an antiviral agent, than ara-A and ara-AMP.[64] Figure 4 shows that the rate of disappearance from mouse plasma of conjugated ara-AMP, as identified by a radioimmunologic method (RIA), was equal to that of L_{30}-HSA-bound radioactivity after administration of an equal dose of the conjugate radioactive in the drug moiety.[63] Since the RIA does not detect coupled ara-hypoxanthine-monophosphate,[63] this result demonstrated that, in contrast to the free drug, ara-AMP bound to L_{30}-HSA was not deaminated in mouse plasma. The resistance of coupled ara-AMP to blood enzymes was probably a necessary condition for the conjugate to be pharmacologically active in liver (see below). The vector L-SA could introduce the aminated and active form of the drug into hepatocytes, although it remained for a relatively long time in the bloodstream before its liver uptake was completed (Figures 3, 4, and 6).

As shown by Table 3, the conjugate interacted in vivo with the hepatic receptor for galactosyl-terminating glycoproteins; it inhibited the disappearance of [^{14}C] AF from the blood of mice to the same extent as did the nonconjugated carrier.[58]

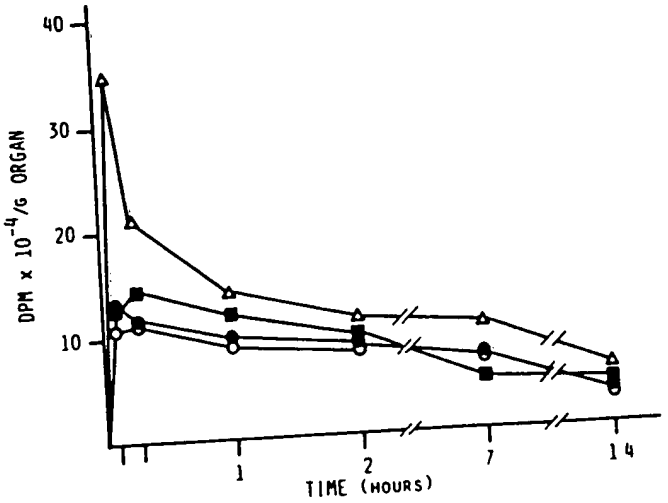


FIGURE 5. Organ distribution of radioactivity in mouse after i.v. injection of free ara-[2,8-^{3}H] AMP (7.5 μg/g) (specific activity 1.4 × 10^4 dpm/μg). (●) Liver; (△) kidneys; (■) spleen; (○) intestine. Each entry represents the mean value of results from four animals. S.E. ranged from 2 to 12% of mean values. (From Fiume, L., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G., *Biochem. Pharmacol.*, 35, 967, 1986. With permission.)

Figures 5 and 6 show organ distribution of radioactivity in mice after i.v. injection of free and coupled ara -[2.8^3-H] AMP. In animals injected with the free drug, acid-soluble radioactivity was equally distributed in liver, spleen, and intestine; higher values were found in kidney. In mice administered with the conjugate, the values of both acid-soluble and acid-insoluble radioactivities were much higher in liver than in the other organs.[58] Also in rats injected i.v. with the radioactive conjugate, both acid-soluble and acid-insoluble radioactivities were several times higher in liver than in intestine, spleen, and kidney.[65] The distribution of the conjugate in parenchymal and sinusoidal cells of rat liver isolated by collagenase perfusion according to Seglen[66] was measured and compared to that of the carrier L_{30}-HSA, AF, HSA, and a complex of ara-AMP with HSA[65] (Table 4). These experiments were performed in order to evaluate the extent of the conjugate uptake by hepatocytes. The concentration of L_{30}-HSA resulted seven times higher in parenchymal than in sinusoidal cells; on the contrary the concentration of AF was equal in the two cell types.* Conjugation with ara-AMP increased the uptake of L_{30}-HSA by sinusoidal cells, but did not decrease that by hepatocytes. The concentration of L_{30}-[^{3}H]HSA-ara-AMP_{13} was practically equal in both cell types. This finding showed that the high uptake of the conjugate by whole liver was not due mainly to sinusoidal cells, but also corresponded to a high conjugate concentration in hepatocytes, which are the liver cells where ara-AMP should be targeted in chronic hepatitis B treatment. In hepatic cells, ara-AMP was released from the conjugate in a pharmacologically active form; administered to mice with Ectromelia virus hepatitis, this conjugate inhibited DNA synthesis in liver without producing significant inhibition in intestine and bone marrow (Figure 7). The liver inhibition produced by coupled ara-AMP at a dose of 3 μg/g reached the same level (40 to 50%) as that brought about by the free drug injected at a dose of 7.5 μg/g, which corresponds to that administered to patients with chronic hepatitis B (5 to 10 mg/kg). Moreover, the inhibitory effect of coupled ara-AMP lasted for a longer period (4 to 5 hr) than that of the free drug (1 hr) (Figure 7).

* Taking into account that hepatocyte volume is about 20 times higher than that of nonparenchymal cells,[67] the uptake of AF by hepatocytes is severalfold higher than that by nonsinusoidal cells.

Table 3
EFFECT OF L_{30}-HSA-ara-AMP_{14} ON PLASMA DISAPPEARANCE OF [^{14}C]AF

Compound injected with [^{14}C]AF	Plasma (dpm/200 μℓ)
None	3,658 ± 621
HSA	3,520 ± 501
AF	9,106 ± 1,022
L_{30}-HSA	9,470 ± 1,370
L_{30}-HSA-ara-AMP_{14}	11,185 ± 1,105

Note: Fetuin was enzymatically desialylated. AF was labeled with [^{14}C] formaldehyde. Femal Swiss mice weighing 28 to 30 g were injected i.v. with 2 μg/g[^{14}C]AF (4.9×10^6 dpm/mg). HSA, AF, L_{30}-HSA, and the conjugate were administered i.v. simultaneously with [^{14}C]AF at 2 μg/g body wt. In all cases, the volume injected was 10 μℓ/g. After 10 min, animals were bled from retroorbital plexus under ether anesthesia, and the radioactivity of plasma was measured. Each entry represents the mean value ± S.E. of results from 10 animals.

From Fiume, L., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G., *Biochem. Pharmacol.*, 35, 967, 1986. With permission.

The conjugate appears to be devoid of acute toxicity in mouse,[58] rat, and rabbit.[68] When administered i.v. at 1 to 2 mg/g body weight, it did not produce any recognizable sign of toxicity in these animals. These were the highest doses tested and are about 25 to 50 times higher than those effective in inhibiting virus DNA synthesis in liver of Ectromelia virus-infected mice. Also, mice injected i.p. with a L_{30}-M(mouse)SA-ara-AMP_{14} for 6 days/week for 4 consecutive weeks (single daily dose = 50 μg/g) did not display any sign of toxicity. The increase in the body weight as well as the food intake per day were equal to those of control mice. No change was observed in liver and kidneys of treated animals at light and electron microscope.[69]

As far as the immunogenicity of type II conjugate is concerned, L_{30}-MSA-ara-AMP_{12-14} administered i.p. to mice for 5 days/week for 4 consecutive weeks (single daily dose = 35 μg/g) raised antibodies in low titers in half of treated animals (the antigen-binding capacity of positive sera ranged from 7 to 104 pmol antigen per milliliter). The same conjugate injected i.v. according to the schedule followed for the intraperitoneal (i.p.) treatment did not produce antibodies in any mouse.[69] Provided that the same immunological behavior is true in man, type II conjugate of ara-AMP with L_{30}-HSA should not induce antibody production when administered i.v. to patients with chronic hepatitis B.

There are human plasma glycoproteins, such as α-acid-glycoprotein, which after desialylation and consequent exposure of galactosyl residues, are selectively taken up by hepatocytes.[17,18] Conjugates of ara-AMP with these glycoproteins might be nonimmunogenic in man. However, the plasma content of these proteins is relatively scarce and, consequently, in contrast to L_{30}-HSA, they can hardly be obtained in the amounts required for clinical purposes.

2. Conjugate with Acyclovir

9-(1-Hydroxyethoxymethyl) guanine [acyclovir (ACV)], a highly potent antiviral drug in herpes viruses diseases,[70,71] has been used in patients with chronic hepatitis B infection with encouraging results.[72,73] However, ACV causes side effects, the most dangerous of which is its deposition in the kidneys due to its limited solubility in urine.[71] Liver targeting of ACV should decrease the risk of this adverse reaction. ACV was converted to its mono-

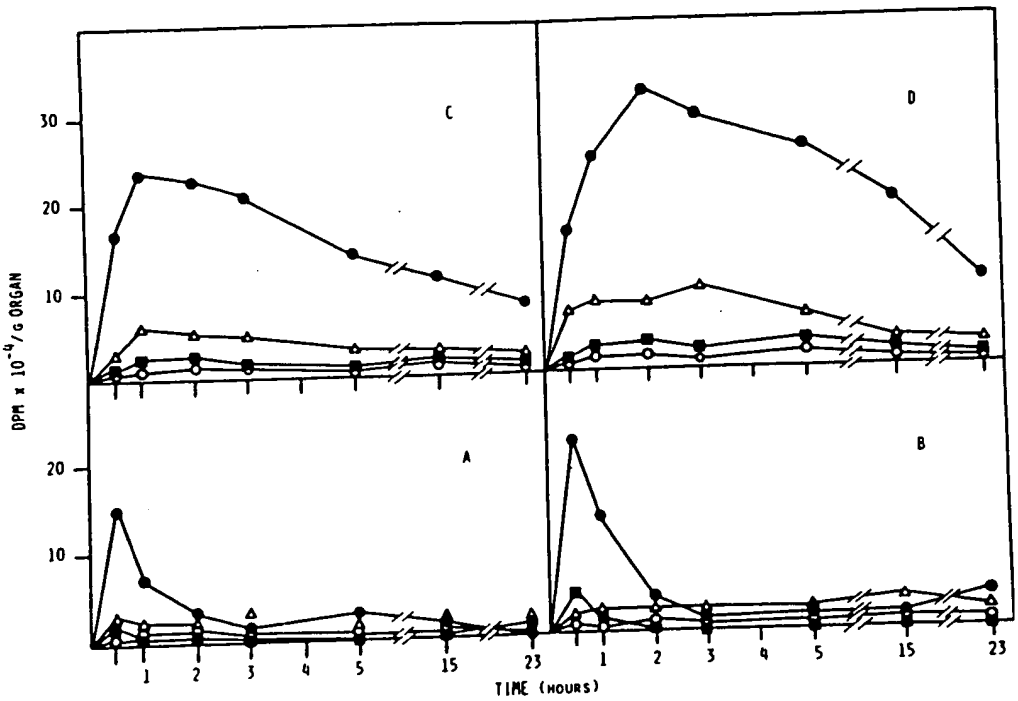


FIGURE 6. Organ distribution of acid-insoluble (frames A and B) and acid-soluble (frames C and D) radioactivities after injection of coupled ara-[2,8-^{3}H]AMP (specific activity 1.4 × 10^4 dpm/μg) at doses of 2 μg/g (frames A and C) or 3 μg/g (frames B and D). (●) Liver; (△) kidneys; (■) spleen; (○) intestine. Each entry represents the mean value of results from four animals. S.E. ranged from 3 to 21% of mean values. (From Fiume, L., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G., *Biochem. Pharmacol.*, 35, 967, 1986. With permission.)

Table 4
DISTRIBUTION OF DIFFERENT PROTEINS AND PROTEIN-ara-AMP CONJUGATES IN PARENCHYMAL AND SINUSOIDAL CELLS OF RAT LIVER 30 MIN AFTER I.V. INJECTION

Compound	Parenchymal cells	Sinusoidal cells
[^{3}H]HSA	1.6 ± 0.9	5.01 ± 2.1
[^{3}H]AF	10.1 ± 2.6	11.6 ± 1.6
L_{30}-[^{3}H]HSA	15.3 ± 2.5	2.0 ± 0.6
[^{3}H]HSA-ara-AMP_{13}	4.6 ± 1.1	52.4 ± 5.2
L_{30}-[^{3}H]HSA-ara-AMP_{14}	13.8 ± 0.1	15.1 ± 0.3

Note: Data were calculated from acid-insoluble radioactivity and related to cell protein; they were expressed as percentage of injected dpm × 10^4/mg protein. Each entry represents the mean (±S.E.) of results from 3 to 4 animals.

phosphate (ACV-MP), which was subsequently conjugated with L_{30}-HSA[74] by the coupling procedure followed for preparing type II conjugate of ara-AMP.[58] The molar ratio of drug to carrier was 10. Table 5 shows the effects of free ACV, free ACV-MP, and the conjugate on DNA synthesis in liver, intestine, and bone marrow of Ectromelia virus-infected mice. ACV and ACV-MP were not active at a dose of 5 μg/g body weight. ACV significantly

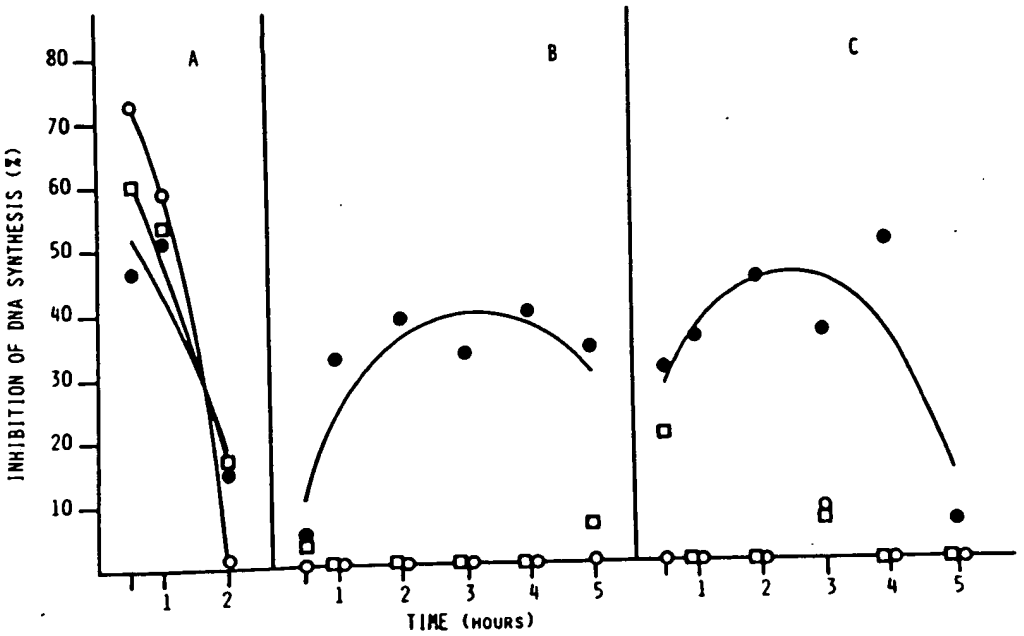


FIGURE 7. Effect of free and coupled ara-AMP on [methyl-^{3}H]thymidine incorporation into DNA in liver (●), intestine (○), and bone marrow (□) of Ectromelia virus-infected mice. Free ara-AMP (frame A) was injected i.v. at a dose of 7.5 μg/g; coupled ara-AMP was injected at doses of 2 μg/g (frame B) or 3 μg/g (frame C). Mice were killed 30 min after i.p. injection of [methyl-^{3}H] thymidine (specific activity 25 Ci/mmol) (0.7 μCi/g body weight). In control animals, the mean values of dpm/100 μg DNA ranged in the different experiments from 23,649 to 31,120 in liver; from 43,474 to 52,340 in intestine; and from 19,284 to 24,331 in bone marrow. Data have been evaluated by means of the analysis of variances. When the interaction time by treatment was found to be significant, the regression lines have been calculated by the method of least squares. For each time, the percentages of inhibition experimentally determined are indicated by the symbols. Coupled ara-AMP was never found to cause a significant inhibition in intestine or bone marrow as evaluated by Student's *t*-test. (From Fiume, L., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G., *Biochem. Pharmacol.*, 35, 967, 1986. With permission.)

inhibited DNA synthesis in liver and intestine at a dose of 10 μg/g. ACV-MP conjugated with L_{30}-HSA inhibited DNA synthesis in liver at a dose as low as 0.6 μg/g without producing singificant inhibition in intestine and bone marrow. These results indicate that after administration of L_{30}-HSA-ACV-MP_{10}, the drug was concentrated in liver in a pharmacologically active form.

In these experiments, coupled ACV-MP inhibited DNA synthesis in liver at doses 10 to 20 times lower than free ACV (Table 5). Besides the selective delivery of the coupled drug to liver cells, another mechanism possibly accounts for this increase in activity. The antiviral activity of ACV depends on the compound being phosphorylated to triphosphate. Phosphorylation to monophosphate is efficiently performed by a virus-coded kinase which is present in herpes virus-infected cells.[75] The monophosphate is further converted to ACV triphosphate by cellular enzymes. In the absence of herpes virus kinase, phosphorylation of ACV is restricted in mammalian cells and consequently the activity of the drug against other viruses is relatively low. Injected ACV-MP did not display a higher activity than ACV (Table 5) presumable because nucleotides very scarcely enter into the cells[76] which they can efficiently penetrate only after dephosphorylation.[77-79] After the uptake of L_{30}-HSA-ACV-MP_{10} by liver cells, the drug which is released from the conjugate in lysosomes may be ACV, ACV-MP, or both (see Section III.A.2). If ACV-MP is set free and can cross the lysosomal membrane, the limiting step of enzymatic phosphorylation of ACV will be bypassed in liver by the intracellular transport of the chemically phosphorylated drug carried by L_{30}-HSA. Studies on the toxicity and immunogenicity of ACV-MP conjugate have not yet been performed.

Table 5
EFFECT OF FREE AND COUPLED ara-AMP AND ACV ON [METHYL-^{3}H] THYMIDINE INCORPORATION INTO DNA OF LIVER, INTESTINE, AND BONE MARROW OF ECTROMELIA VIRUS-INFECTED MICE[a]

Compound	Dose (μg/g)	ara-AMP or ACV administered (μg/g)	Inhibition of [methyl-^{3}H] thymidine incorporation (%)		
			Liver	Intestine	Bone marrow
ara-AMP	7.5	7.5	49(S)[b]	61(S)	34(S)
L_{30}-HSA-ara-AMP_{13}	50	3.2	50(S)	6(NS)[b]	9(NS)
ACV	5	5	0	7	0
	10	10	42(S)	38(S)	15(NS)
	100	100	65(S)	75(S)	59(S)
ACV-MP	6.8	5	15(NS)	23(NS)	20(NS)
L_{30}-HSA-ACV-MP_{10}	25	0.6	40(S)	6(NS)	0
	50	1.2	55(S)	16(NS)	7(NS)

[a] Compounds were injected 1 hr before [methyl-^{3}H] thymidine. In control animals, the mean values of dpm/100 μg DNA ranged in the different experiments from 14,177 to 24,692 in liver; from 19,141 to 24,121 in intestine; and from 16,967 to 21,289 in bone marrow.

[b] Results were statistically evaluated by means of Student's *t*-test. The difference was considered statistically significant (S) or not significant (NS) for $p <$ or > 0.05, respectively.

C. Galactosylated Poly(L-Lysine) Conjugate

Poly(L-lysine) has been used by several authors as a drug carrier in experiments of cancer chemotherapy in animals (for review, see Arnold[80]). This polymer (M_r 47,000) was conjugated[74] with 2-imino-2-methoxyethyl 1-thioglycoside of D-galactose prepared according to Lee et al.[81] The resulting complex [gal-poly(L-lysine)] had about one third of the lysine residues substituted by the sugar groups and in mice it bound to the hepatic receptor for galactosyl-terminating glycoproteins. ara-AMP was coupled to this galactosylated poly(L-lysine) by means of ECDI at pH 7.5. The weight ratio of drug to carrier of the complex was 0.18. In mice with Ectromelia virus hepatitis, gal-poly(L-lysine)-ara-AMP accomplished a liver targeting of the drug.[74] However, the toxicity of poly(L-lysine),[80,82] although reduced after glycosylation, remained very high (in mouse, LD_{50} of gal-poly(L-lysine)-ara-AMP = 40 μg/g body weight[74]), thus precluding the possibility of a clinical use of this conjugate.

IV. ANTIBODY CONJUGATE

Interferon α/β (IFN) was coupled by means of *N*-succinimidyl-3-(2-pyrridyldithio)propionate to a monoclonal antibody (MAb) specific for an Epstein-Barr virus (EBV) membrane antigen (MA).[83] The molar ratio of IFN to MAb in the conjugate was very low (10^{-3} to 10^{-4}). The effect of free and coupled IFN on Mengo virus growing in human tumor cell lines QIMR-WIL (EBV-MA$^+$) and U-266 (EBV-MA$^-$) was studied. When IFNs were present in the culture medium during the period of virus growth, no difference between the antiviral activities of free and coupled drug was observed. In the experiments in which the cells were pulsed with IFNs for 15 min at 4°C and then repeatedly washed, the antiviral activity of IFN-MAb was higher than that of noncoupled IFN. The higher antiviral activity of IFN-MAb was observed only in the cells possessing the EBV-MA.

These experiments demonstrate that the biological activity of IFN is maintained after coupling to antibodies, but the selective delivery to cells possessing the antigen specific for the conjugated MAb was obtained under conditions that cannot be accomplished in vivo.

In fact, a higher activity of IFN-MAb on EBV-MA$^+$ cells was found only when the cells were pulsed for 15 min with free or coupled drug at 4°C; under these conditions, there was no binding of IFN to its receptor, while antibodies could link to the specific antigen.[83,84]

V. HORSERADISH PEROXIDASE CONJUGATE

There is evidence that herpes simplex virus (HSV) infections of the cornea recur because the trigeminal ganglia harbor latent virus during periods of remission of active corneal disease.[85] An attempt was made[86] to deliver antiviral agents selectively to neuronal cells in the trigeminal ganglion by coupling to horseradish peroxidase (HRP), a protein which travels from cornea to ganglion somata by a process of retrograde axonal transport.[87] 5′-Iodo-5′-amino-2′,5′-dideoxyuridine (AIDU), a powerful antiviral agent against herpes simplex,[88] was synthesized using ^{125}I and conjugated with HRP. Coupling was performed by a Schiff base formation between the 5′ amino group of the drug and aldehydes generated on the carbohydrate moiety of HRP by means of a short periodate oxidation. Preparations of the AIDU-HRP conjugate with molar ratios of drug to protein ranging from 2 to 9 were used in the experiments in vivo. The conjugate was injected into the corneal stroma of rabbits, and the delivery of the coupled drug to trigeminal ganglion cells was demonstrated by the radioactivity detected in these cells by means of autoradiography.[86] The presence of the radioactive drug in trigeminal ganglion somata was due to the effect of HRP moving by retrograde axonal transport. In fact, after the corneal stroma was injected with similar concentrations of the free radioactive drug or of radioactive drug simply mixed with HRP, trigeminal ganglion cells were not labeled. In these experiments, however, no evidence was obtained as to whether or not the drug was released into the target cells in a pharmacologically active form.

VI. CONCLUSIONS

Among the protein conjugates with antiviral drugs prepared so far, only that of L-SA with ara-AMP (type II) was found, at least in mouse, to fulfill the criteria required by a drug-carrier complex (see Section I). L-SA-ara-AMP is stable in the bloodstream where the drug is neither released nor deaminated; it maintains the capacity of lactosaminated albumin to interact with the specific receptors on the surface of hepatocytes with which it can freely come in contact through the hepatic sinusoids; it is selectively taken up by the liver where the albumin carrier is digested. The released drug remains in high concentration in liver and its pharmacological action is confined to this organ. Moreover, this conjugate does not display any recognizable sign of acute toxicity even at doses several fold higher than those pharmacologically active and, when prepared with homologous albumin and injected i.v., is not immunogenic. Finally, a great practical advantage of type II L-SA-ara-AMP conjugate is its chemical stability; in contrast to type I complex, it remains soluble after lyophilization. If this conjugate behaves in man as in mouse, and HBV-infected hepatocytes do not lose the receptor for galactosyl-terminating glycoproteins, its administration to patients with chronic hepatitis B should result in a selective delivery of ara-AMP to liver with the following consequences: (1) a more efficient inhibition of virus replication in this organ; (2) a lower toxicity for the other tissues, permitting prolongation of the antiviral treatment; and (3) less damage to lymphocytes that were reported to be injured by ara-A,[89] with a consequent better cooperation of the immune response. Altogether these effects could facilitate the clearance of virus from liver, which in turn should allow the specific immune lymphocytes to address their action only to the extrahepatic cells which might also be infected,[90-93] thus increasing the probability of their elimination.

NOTE ADDED IN PROOFS

After the manuscript was submitted to the editor, four volunteers (HBsAg, HBeAg, HBV-DNA positive for more than 6 months) received an L_{30}-HSA-ara-AMP_{10} conjugate (type II) at the Gastroenterology Department of Prof. Verme (Turin). The conjugate (prepared by Laboratori Baldacci, Pisa) was given by 0.5 hr intravenous infusion for 3 consecutive days at the daily dose of 35 mg/kg corresponding to 1.5 mg ara-AMP/kg. In three patients serum HBV-DNA, measured by dot-blot hybridization, dropped from 50 to 500 pg/mℓ to undetectable levels (<0.1 pg/mℓ). In the remaining patient, HBV-DNA became undetectable after a second conjugate course. No side effects were observed; blood chemistry and transaminases remained at the pretreatment levels.

These results indicate that L-HSA-ara-AMP inhibits HBV replication and prompt further studies to define the minimal effective dose for prolonged treatment.

ACKNOWLEDGMENTS

Authors' work reported in this review was supported by Consiglio Nazionale delle Ricerche, Progetti Finalizzati Virus, Controllo Crescita Neoplastica, Oncologia (grants 84.00578.44, 85.02157.44, and 86.00411.44); Ministero Pubblica Istruzione Italiano; and Associazione Italiana per la Ricerca sul Cancro. The authors wish to thank Prof. G. Prodi for his continued support and encouragement to their research.

REFERENCES

1. **Trouet, A., Baurain, R., Deprez-De Campeenere, D., Masquelier, M., and Pirson, P.,** Targeting of antitumour and antiprotozoal drugs by covalent linkage to protein carriers, in *Targeting of Drugs*, Vol. 47, Gregoriadis, G., Senior, J., and Trouet, A., Eds., Nato Advanced Studies Institutes Series A, Plenum Press, New York, 1982, 1.
2. **Balboni, P. G., Minia, A., Grossi M. P., Barbanti-Brodano, G., Mattioli, A., and Fiume, L.,** Activity of albumin conjugates of 5-fluorodeoxyuridine and cytosine arabinoside on poxviruses as a lysosomotropic approach to antiviral chemotherapy, *Nature (London)*, 264, 181, 1976.
3. **Cessi, C. and Fiume, L.,** Increased toxicity of β-amanitin when bound to a protein, *Toxicon*, 6, 309, 1969.
4. **Barbanti-Brodano, G. and Fiume, L.,** Selective killing of macrophages by amanitin-albumin conjugates, *Nature (London), New Biol.*, 243, 281, 1973.
5. **Fiume, L. and Barbanti-Brodano, G.,** Selective toxicity of amanitin-albumin conjugates for macrophages, *Experientia*, 30, 76, 1974.
6. **Derenzini, M., Fiume, L., Marinozzi, V., Mattioli, A., Montanaro, L., and Sperti, S.,** Pathogenesis of liver necrosis produced by amanitin-albumin conjugates, *Lab. Invest.*, 29, 150, 1973.
7. **Bonetti, E., Derenzini, M., and Fiume, L.,** Lesions in the cells of proximal convoluted tubules in rat kidney induced by amanitin-albumin conjugate, *Virchows Arch. B*, 16, 71, 1974.
8. **Marinozzi, V. and Fiume, L.,** Effects of α-amanitin on mouse and rat liver cell nuclei, *Exp. Cell Res.*, 67, 311, 1971.
9. **Barbanti-Brodano, G., Derenzini, M., and Fiume, L.,** Toxic action of phalloidin-albumin conjugate on cells with a high protein uptake, *Nature (London)*, 248, 63, 1974.
10. **Van Furth, R., Cohn, Z. A., Hirsch, J. G., Humphrey, J. H., Spector, W. G., and Langevoort, H. L.,** The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells, *Bull. World Health Org.*, 46, 845, 1972.
11. **Barbanti-Brodano, G. and Fiume, L.,** In vitro effect of a 5-fluorodeoxyuridine albumin conjugate on tumor cells and on peritoneal macrophages, *Experientia*, 30, 1180, 1974.
12. **Roy-Burman, P.,** *Analogues of Nucleic Acid Components. Recent Results in Cancer Research*, Vol. 25, Springer-Verlag, New York, 1970, chap. 3.
13. **Halloran, M. J. and Parker, C. W.,** The preparation of nucleotide protein conjugates: carbodiimides as coupling agents, *J. Immunol.*, 96, 373, 1966.

14. **Mims, C. A.,** The response of mice to large intravenous injection of Ectromelia virus. II. The growth of virus in the liver, *Br. J. Exp. Pathol.*, 40, 543, 1959.
15. **Roberts, J. A.,** Histopathogenesis of mousepox: Ectromelia virulence, *Br. J. Exp. Pathol.*, 44, 465, 1963.
16. **Fiume, L., Mattioli, A., Balboni, P. G., Tognon, M., Barbanti-Brodano, G., De Vries, J., and Wieland, Th.,** Enhanced inhibition of virus DNA synthesis in hepatocytes by trifluorothymidine coupled to asialofetuin, *FEBS Lett.*, 103, 47, 1979.
17. **Ashwell, G. and Morell, A. G.,** The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins, *Adv. Enzymol.*, 41, 99, 1974.
18. **Ashwell, G. and Harford, J.,** Carbohydrate-specific receptors of the liver, *Annu. Rev. Biochem.*, 51, 531, 1982.
19. **Rogers, J. C. and Kornfeld, S.,** Hepatic uptake of proteins coupled to fetuin glycopeptide, *Biochem. Biophys. Res. Commun.*, 45, 622, 1971.
20. **Gregoriadis, G. and Neerunjun, E. D.,** Homing of liposomes to target cells, *Biochem. Biophys. Res. Commun.*, 65, 537, 1975.
21. **Heidelberger, C. and King, D. H.,** Trifluorothymidine, *Pharmacol. Ther.*, 6, 427, 1979.
22. **Edwards, J. L. and Klein, R. E.,** Cell renewal in adult mouse tissues, *Am. J. Pathol.*, 38, 437, 1961.
23. **Fiume, L., Busi, C., Mattioli, A., Balboni, P. G., and Barbanti-Brodano, G.,** Hepatocyte targeting of adenine-9-β-D-arabinofuranoside 5′-monophosphate (ara-AMP) coupled to lactosaminated albumin, *FEBS Lett.*, 129, 261, 1981.
24. **Fiume, L., Mattioli, A., Busi, C., Balboni, P. G., Barbanti-Brodano, G., De Vries, J., Altmann, R., and Wieland, Th.,** Selective inhibition of Ectromelia virus DNA synthesis in hepatocytes by adenine-9-β-D-arabinofuranoside (ara-A) and adenine-9-β-D-arabinofuranoside 5′-monophosphate (ara-AMP) coupled to asialofetuin, *FEBS Lett.*, 116, 185, 1980.
25. **Chadwick, R. G., Bassendine, M. F., Crawford, E. M., Thomas, H. C., and Sherlock, S.,** HBsAg-positive chronic liver disease: inhibition of DNA polymerase activity by vidarabine, *Br. Med. J.*, 2, 531, 1978.
26. **Pollard, R. B., Smith, J. L., Neal, E. A., Gregory, P. B., Merigan, T. C., and Robinson, W. S.,** Effect of vidarabine on chronic hepatitis B virus infection, *JAMA*, 239, 1648, 1978.
27. **Preiksaitis, J. K., Lank, B., Ng, P. K., Brox, L., Le Page, G. A., and Tyrrell, D. L. J.,** Effect of liver disease on pharmacokinetics and toxicity of 9-β-D-arabinofuranosyladenine-5-phosphate, *J. Infect. Dis.*, 144, 358, 1981.
28. **Bassendine, M. F., Chadwick, R. G., Salmeron, J., Shipton, U., Thomas, H. C., and Sherlock, S.,** Adenine arabinoside therapy in HBsAg-positive chronic liver disease: a controlled study, *Gastroenterology*, 80, 1016, 1981.
29. **Scullard, G. H., Pollard, R. B., Smith, J. L., Sacks, S. L., Gregory, P. B., Robinson, W. S., and Merigan, T. C.,** Antiviral treatment of chronic hepatitis B virus infection. I. Changes in viral markers with interferon combined with adenine arabinoside, *J. Infect. Dis.*, 143, 772, 1981.
30. **Scullard, G. H., Andres, L. L., Greenberg, H. B., Smith, J. L., Sawhney, V. K., Neal, E. A., Mahal, A. S., Popper, H., Merigan, T. C., Robinson, W. S., and Gregory, P. B.,** Antiviral treatment of chronic hepatitis B virus infection: improvement in liver disease with interferon and adenine arabinoside, *Hepatology*, 1, 228, 1981.
31. **Scullard, G. H., Greenberg, H. B., Smith, J. L., Gregory, P. B., Merigan, T. C., and Robinson, W. S.,** Antiviral treatment of chronic hepatitis B infection. II. Infectious virus cannot be detected in patient serum after permanent responses to treatment, *Hepatology*, 2, 39, 1982.
32. **Weller, I. V. D., Bassendine, M. F., Craxi, A., Fowler, M. J. F., Monjardino, J., Thomas, H. C., and Sherlock, S.,** Successful treatment of HBs and HBeAg positive chronic liver disease: prolonged inhibition of viral replication of highly soluble adenine arabinoside 5′-monophosphate (ara-AMP), *Gut*, 23, 717, 1982.
33. **Smith, C. I., Kitchen, L. W., Scullard, G. H., Robinson, W. S., Gregory, P. B., and Merigan, T. C.,** Vidarabine monophosphate and human leukocyte interferon in chronic hepatitis B infection, *JAMA*, 247, 2261, 1982.
34. **Hoofnagle, J. H., Minuk, G. Y., Dusheiko, G. M., Schafer, D. F., Johnson, R., Straus, S., Jones, E. A., Gerin, J. L., and Ishak, K.,** Adenine arabinoside 5′-monophosphate treatment of chronic type B hepatitis, *Hepatology*, 2, 784, 1982.
35. **Hoofnagle, J. H., Hanson, R. G., Minuk, G. Y., Pappas, S. C., Schafer, D. F., Dusheiko, G. M., Strauss, S. E., Popper, H., and Jones, E. A.,** Randomised controlled trial of adenine arabinoside monophosphate for chronic type B hepatitis, *Gastroenterology*, 86, 150, 1984.
36. **Hoofnagle, J. H., Davis, G. L., Hanson, R. G., Pappas, S. C., Peters, M. G., Avigan, M. I., Waggoner, J. G., Howard, R., Jones, E. A., and Straus, S. E.,** Treatment of chronic type B hepatitis with multiple ten-day courses of adenine arabinoside monophosphate, *J. Med. Virol.*, 15, 121, 1985.

37. **Perrillo, R. P., Regenstein, F. G., Bodicky, C. J., Campbell, C. R., Sanders, G. E., and Sunwoo, J. C.,** Comparative efficacy of adenine arabinoside 5′-monophosphate and prednisone withdrawal followed by adenine arabinoside 5′-monophosphate in the treatment of chronic active hepatitis type B, *Gastroenterology*, 88, 780, 1985.
38. **Yokosuka, O., Omata, M., Imazeki, F., Hirota, K., Mori, J., Uchiumi, K., Ito, Y., and Okuda, K.,** Combination of short-term prednisolone and adenine arabinoside in the treatment of chronic hepatitis B, *Gastroenterology*, 89, 246, 1985.
39. **Loeschke, K., Abb, J., Farack, U. M., and Wiebecke, B.,** Treatment of HBsAg-HBcAg-positive chronic active hepatitis with adenine arabinoside. A case report with clinical remission and seroconversion to anti-HBs, *Hepatogastroenterology*, 32, 178, 1985.
40. **Weller, I. V. D., Lok, A. S. F., Mindel, A., Karayannis, P., Galpin, S., Monjardino, J., Sherlock, S., and Thomas, H. C.,** Randomised controlled trial of adenine arabinoside 5′-monophosphate (ara-AMP) in chronic hepatitis B virus infection, *Gut*, 26, 745, 1985.
41. **Lok, A. S. F., Novick, D. M., Karayannis, P., Dunk, A. A., Sherlock S., and Thomas, H. C.,** A randomized study of the effects of adenine arabinoside 5′-monophosphate (short or long courses) and lymphoblastoid interferon on hepatitis B virus replication, *Hepatology*, 6, 1132, 1985.
42. **Sacks, S. L., Smith, J. L., Pollard, R. B., Sawhney, V., Mahol, A. S., Gregory, P., Merigan, T. C., and Robinson, W. S.,** Toxicity of vidarabine, *JAMA*, 241, 28, 1979.
43. **Sacks, S. L., Scullard, G. H., Pollard, R. B., Gregory, P. B., Robinson, W. S., and Merigan, T. C.,** Antiviral treatment of chronic hepatitis B virus infection: pharmacokinetics and side effects of interferon and adenine arabinoside alone and in combination, *Antimicrob. Agents Chemother.*, 21, 93, 1982.
44. **Whitley, R., Alford, C., Hess, F., and Buchanan, R.,** Vidarabine: a preliminary review of its pharmacological properties and therapeutic use, *Drugs*, 20, 267, 1980.
45. **Lok, A. S. F., Wilson, L. A., and Thomas, H. C.,** Neurotoxicity associated with adenine arabinoside monophosphate in the treatment of chronic hepatitis B virus infection, *J. Antimicrob. Chemother.*, 14, 93, 1984.
46. **Fiume, L., Mattioli, A., Busi, C., Spinosa, G., and Wieland, Th.,** Conjugates of adenine 9-β-D-arabinofuranoside monophosphate (ara-AMP) with lactosaminated homologous albumin are not immunogenic in the mouse, *Experientia*, 38, 1087, 1982.
47. **Fiume, L.,** Penetration of β-amanitin-rabbit-albumin conjugate into hepatic parenchymal cells, *Lancet*, 2, 853, 1969.
48. **Wilson, G.,** Effect of reductive lactosamination on the hepatic uptake of bovine pancreatic ribonuclease A dimer, *J. Biol. Chem.*, 253, 2070, 1978.
49. **Wilson, G., Eidelberg, M., and Michalak, V.,** Selective hepatic uptake of synthetic glycoproteins, *J. Gen. Physiol.*, 74, 495, 1979.
50. **Youle, R. I., Murray, G. J., and Neville, D. M.,** Ricin linked to monophosphopentamannose binds to fibroblast lysosomal hydrolase receptors, resulting in a cell-type-specific toxin, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 5559, 1979.
51. **Krantz, M. J., Holtzman, N. A., Stowell, C. P., and Lee, Y. C.,** Attachment of thioglycosides to proteins: enhancement of liver membrane binding, *Biochemistry*, 15, 3963, 1976.
52. **Gray, G. R.,** The direct coupling of oligosaccharides to proteins and derivatized gels, *Arch. Biochem. Biophys.*, 163, 426, 1974.
53. **Schwartz, B. A. and Gray, G. R.,** Proteins containing reductively aminated disaccharides. Synthesis and chemical characterization, *Arch. Biochem. Biophys.*, 181, 542, 1977.
54. **Fiume, L., Mattioli, A., Busi, C., and Accorsi, C.,** Selective penetration and pharmacological activity of lactosaminated albumin conjugates of adenine arabinoside 5′-monophosphate (ara-AMP) in mouse liver, *Gut*, 25, 1392, 1984.
55. **Fiume, L., Bassi, B., Busi, C., and Mattioli, A.,** Preparation of lactosaminated albumin-ara-AMP conjugate which remains soluble after lyophilization, *Pharm. Acta Helv.*, 60, 318, 1985.
56. **Fiume, L., Bassi, B., and Bongini, A.,** Conjugates of 9-β-D-arabinofuranosyladenine 5′-monophosphate (ara-AMP) with lactosaminated albumin. Characterization of the drug-carrier bonds, *Pharm. Acta Helv.*, in press.
57. **Di Sabato, G. and Jencks, W. P.,** Mechanism and catalysis of reactions of acyl phosphates. I. Nucleophilic reactions, *J. Am. Chem. Soc.*, 83, 4393, 1961.
58. **Fiume, L., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G.,** Drug targeting in antiviral chemotherapy. A chemically stable conjugate of 9-β-D-arabinofuranosyl-adenine 5′-monophosphate with lactosaminated albumin accomplishes a selective delivery of the drug to liver cells, *Biochem. Pharmacol.*, 35, 967, 1986.
59. **Peters, T.,** Serum albumin, *Adv. Protein Chem.*, 37, 161, 1985.
60. **Sheehan, J. C. and Hlavka, J. J.,** The cross-linking of gelatin using a water-soluble carbodiimide, *J. Am. Chem. Soc.*, 79, 4528, 1957.

61. **Jentof, N. and Dearborn, D. G.,** Protein labelling by reductive alkylation, *Methods Enzymol.*, 91, 570, 1983.
62. **Whitley, R. J., Tucker, B. C., Kinkel, A. W., Barton, N. H., Pass, R. F., Whelchel, J. D., Cobbs, C. G., Diethelm, A. G., and Buchanan, R. A.,** Pharmacology, tolerance, and antiviral activity of vidarabine monophosphate in humans, *Antimicrob. Agents Chemother.*, 19, 709, 1980.
63. **Fiume, L., Balboni, P. G., Bassi, B., Busi, C., Mattioli, A., and Spinosa, G.,** 9-β-D-arabinofuranosyladenine 5'-monophosphate coupled to lactosaminated albumin is not metabolized in mouse bloodstream, *Pharm. Acta Helv.*, 61, 342, 1986.
64. **Gephart, J. F. and Lerner, A. M.,** Comparison of the effects of arabinosyladenine, arabinosylhypoxanthine and arabinosyladenine 5'-monophosphate against herpes simplex virus, varicella-zoster virus, and cytomegalovirus with their effects on cellular deoxyribonucleic acid synthesis, *Antimicrob. Agents Chemother.*, 19, 170, 1981.
65. **Fiume, L., Mattioli, A., and Spinosa, G.,** Distribution of a conjugate of 9-β-D-arabinofuranosyladenine 5'-monophosphate (ara-AMP) with lactosaminated albumin in parenchymal and sinusoidal cells of rat liver, *Cancer Drug Delivery*, 4, 11, 1987.
66. **Seglen, P. O.,** Preparation of isolated rat liver cells, *Methods Cell Biol.*, 13, 29, 1976.
67. **Greengard, O., Federman, M., and Knox, W. E.,** Cytomorphometry of developing rat liver and its application to enzymic differentiation, *J. Biol. Chem.*, 52, 261, 1972.
68. **Baldacci, M. and Gervasi, G. B.,** personal communication, 1986.
69. **Fiume, L., Busi, C., Preti, P., and Spinosa, G.,** *Cancer Drug Delivery*, in press.
70. **Schaeffer, H. J., Beauchamp, L., de Miranda, P., Elion, G. B., Bauer, D. J., and Collins, P.,** 9-(2-Hydroxyethoxymethyl) guanine activity against viruses of the herpes group, *Nature (London)*, 272, 583, 1978.
71. **Balfour, H. H.,** Acyclovir and other chemotherapy for herpes group viral infections, *Annu. Rev. Med.*, 35, 279, 1984.
72. **Weller, I. V. D., Carreño, V., Fowler, M. J. F., Monjardino, J., Makinen, D., Varghese, Z., Sweny, P., Thomas, H. C., and Sherlock, S.,** Acyclovir in hepatitis B antigen-positive chronic liver disease: inhibition of viral replication and transient renal impairment with i.v. bolus administration, *J. Antimicrob. Chemother.*, 11, 223, 1983.
73. **Schalm, S. W., Heytink, R. A., Van Buuren, H. R., and De Man, R. A.,** Acyclovir enhances the antiviral effect of interferon in chronic hepatitis B, *Lancet*, 2, 358, 1985.
74. **Fiume, L., Bassi, B., Busi, C., Mattioli, A., Spinosa, G., and Faulstich, H.,** Galactosylated poly (L-lysine) as a hepatotropic carrier of 9-β-D-arabinofuranosyladenine 5'-monophosphate, *FEBS Lett.*, 203, 203, 1986.
75. **Elion, G. B., Furman, P. A., Fyfe, J. A., de Miranda, P., Beauchamp, L., and Schaeffer, H. J.,** Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 5716, 1977.
76. **Plunkett, W., Lapi, L., Ortiz, P. J., and Cohen, S. S.,** Penetration of mouse fibroblasts by the 5'-phosphate of 9-β-D-arabinofuranosyladenine and incorporation of the nucleotide into DNA, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 73, 1974.
77. **Leibman, K. C. and Heidelberger C.,** The metabolism of ^{32}P-labeled ribonucleotides in tissue slices and cell suspension, *J. Biol. Chem.*, 216, 823, 1955.
78. **Roll, P. M., Weinfeld, H., Carroll, E., and Brown, G. B.,** The utilization of nucleotides by the mammal. IV. Triply labeled purine nucleotides, *J. Biol. Chem.*, 220, 439, 1956.
79. **Roll, P. M., Weinfeld, H., and Carroll, E.,** The utilization of nucleotides by the mammal. V. Metabolism of pyrimidine nucleotides, *J. Biol. Chem.*, 220, 455, 1956.
80. **Arnold, L. J.,** Polylysine-drug conjugates, *Methods Enzymol.*, 112, 270, 1985.
81. **Lee, Y. C., Stowell, C. P., and Krantz, M. J.,** 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins, *Biochemistry*, 15, 3956, 1976.
82. **Katchalski, E., Sela, M., Silman, H. I., and Berger, A.,** Polyamino acids as protein models, in *The Proteins. Composition, Structure, and Function*, Vol. 2, 2nd ed., Neurath, H., Ed., Academic Press, New York, 1964, 405.
83. **Miescher-Granger, S., Hochkeppel, H. K., Braun, D. G., and Alkan, S. S.,** Biological activities of human recombinant interferon α/β targeted by anti-Epstein-Barr virus monoclonal antibodies, *FEBS Lett.*, 179, 29, 1985.
84. **Alkan, S. S., Miescher-Granger, S., Braun, D. G., and Hochkeppel, H. K.,** Antiviral and antiproliferative effects of interferons delivered via monoclonal antibodies, *J. Interferon Res.*, 4, 355, 1984.
85. **Baringer, J. R. and Swoveland, P.,** Recovery of herpes simplex virus from human trigeminal ganglions, *N. Engl. J. Med.*, 288, 648, 1973.
86. **Haschke, R. H., Ordronneau, J. M., and Bunt, A. H.,** Preparation and retrograde axonal transport of an antiviral drug-horseradish peroxidase conjugate, *J. Neurochem.*, 35, 1431, 1980.

87. **Kristensson, K.,** Retrograde transport of macromolecules in axons, *Annu. Rev. Pharmacol. Toxicol.*, 18, 97, 1978.
88. **Lin, T. S., Neenan, J. P., Cheng, Y. C., and Prusoff, W. H.,** Synthesis and antiviral activity of 5- and 5′-substituted thymidine analogs, *J. Med. Chem.*, 19, 495, 1976.
89. **Hafkin, B., Pollard, R. B., Tiku, M. L., Robinson, W. S., and Merigan, T. C.,** Effects of interferon and adenine arabinoside treatment of hepatitis B virus infection on cellular immune responses, *Antimicrob. Agents Chemother.*, 16 781, 1979.
90. **Romet-Lemonne, J. P., McLane, M. F., Elfassi, E., Haseltine, W. A., Azocar, J., and Essex, M.,** Hepatitis B virus infection in cultured human lymphoblastoid cells, *Science*, 221, 667, 1983.
91. **Blum, H. E., Stowring, L., Figus, A., Montgomery, C. K., Haase, A. T., and Vyas, G. N.,** Detection of hepatitis B virus DNA in hepatocytes, bile duct epithelium, and vascular elements by *in situ* hybridization, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 6685, 1983.
92. **Elfassi, E., Romet-Lemonne, J. L., Essex, M., Frances-McLane, M., and Haseltine, W. A.,** Evidence of extrachromosomal forms of hepatitis B viral DNA in a bone marrow culture obtained from a patient recently infected with hepatitis B virus, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 3526, 1984.
93. **Pontisso, P., Poon, M. C., Tiollais, P., and Brechot, C.,** Detection of hepatitis B virus DNA in mononuclear blood cells, *Br. Med. J.*, 288, 1563, 1984.